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PARAMETERS FOR THE DETECTION OF COLORECTAL CANCER RECURRENCES

PARÂMETROS PARA A DETECÇÃO DE RECIDIVAS DE CANCRO COLORECTAL

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Farmacêutica, realizada sob a orientação científica do Doutor Francisco Pimentel, Professor Associado com Agregação, Convidado da Secção Autónoma de Ciências da Saúde da Universidade de Aveiro e do Doutor Amílcar Falcão, Professor Catedrático da Faculdade de Farmácia da Universidade de Coimbra.

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palavras-chave neoplasias colorectais,marcadores tumorais, antigénio carcinoembrionário, glucose, desidrogenase láctica

resumo

A detecção de recidivas antes da manifestação dos primeiros sintomas é essencial para atingir bons resultados na gestão de doenças neoplásicas. O antigénio carcinoembrionário é o marcador tumoral bioquímico para cancro colorectal mais usado desde há já muitos anos mas a sua variabilidade na detecção de recidivas é elevada. Este trabalho propôs-se a identificar parâmetros que, associados a antigénio carcinoembrionário, melhorem a detecção de recidivas de cancro colorectal entre dados bioquímicos, clínicos e clinico-patológicos de pacientes submetidos a cirurgias de intenção curativa para essa mesma patologia, utilizando registos retrospectivos clínicos diários dos serviços de Cirurgia Geral e Oncologia do Hospital Infante D. Pedro E.P.E em Aveiro.

Recorrendo a análise estatística descritiva tradicional e multivariada, a glucose sérica e o antigénio carcinoembrionário sérico foram identificados como factores de prognóstico de recidivas colorectais. A desidrogenase láctica contribui para o diagnóstico de recidivas quando o antigénio carcinoembrionário não é considerado na análise. Outros dois parâmetros, o antigénio carbohidrato 19-9 e os leucócitos totais foram também relacionados com a ocorrência de recidivas em análise univariada mas perdem valor quando incluídos no modelo multivariado perante a presença do antigénio carcinoembrionário.

Os resultados são apenas indicativos devido à reduzida dimensão da amostra utilizada mas abrem caminho a estudos subsequentes que avaliem estes e outros parâmetros.

keywords colorectal neoplasms, tumor markers, carcinoembryonic antigen, glucose, lactate dehydrogenase.

abstract

The accurate detection of recurrences, prior to the onset of the first clinical symptoms, is essential for achieving favorable results in the management of neoplastic diseases. Carcinoembryonic antigen has been a long-standing biochemical tumor marker for colorectal cancer and is the most widely used in this disease. However, it presents great variability in the detection of recurrences. Henceforth, the identification of additional parameters is of great importance. This work proposed to retrospectively analyze clinical, clinicopathological and biochemical data of patients that had undergone surgical resection of primary colorectal cancer, collected during the daily practice of the Surgery and Oncology Services of the Infante D. Pedro E.P.E Hospital in Aveiro, to identify parameters that, associated with carcinoembryonic antigen, improve the detection of colorectal cancer recurrences

By means of traditional descriptive and multivariant statistical analysis, serum glucose and carcinoembryonic antigen were identified as prognostic factors for colorectal recurrences. Lactate dehydrogenase contributes to the diagnosis of recurrences when carcinoembryonic antigen is no considered in the analysis. Other two parameters, carbohydrate antigen 19-9 and total white blood cell count were also found related with recurrence in univariant analysis but lost significance when compared with carcinoembryonic antigen in the multivariant analysis.

The results are merely indicative due to the reduced size of the patient sample but lay ground for subsequent, more powerful studies.

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ABBREVIATIONS

ALB	Albumin
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
ASCO	American Society of Clinical Oncology
AST	Aspartate Aminotransferase
AUC	Area Under the Curve
CA 19-9	Carbohydrate Antigen
CEA	Carcinoembryonic Antigen
CI	Confidence Interval
CRC	Colorectal Cancer
CRP	C-Reactive Protein
DFS	Disease-free survival
EGFR	Epidermal Growth Factor Receptor
ESMO	European Society for Medical Oncology
ESR	Erythrocyte Sedimentation Rate
Gamma-GT	Gamma-Glutamyltransferase
GI	Gastrointestinal
HR	Hazard Ratio
IDPH	Infante D. Pedro E.P.E Hospital
LDH	Lactate Dehydrogenase
LDL	Cholesterol - LDL
LFTs	Liver Function Tests
LVI	Lymphovascular Invasion
MCHC	Mean Corpuscular Hemoglobin Concentration
NCCN	National Comprehensive Cancer Network
PNI	Perineural Invasion
RDW	Red Blood Cell Distribution Width
ROC	Receiver Operating Characteristic
SE	Standard Error
TNM	Tumor Node Metastasis
TWBC	Total White Blood Cells
UA	University of Aveiro
UICC	*Union Internationale Contre le Cancer*

CHAPTER 1: INTRODUCTION

The scope of this dissertation was to establish the relationship between some biological parameters and the prediction of recurrence of colorectal cancer.

In this chapter, we discuss the three concepts involved: anatomy and histology of the digestive tract, colorectal cancer and tumor markers.

1.1. OVERVIEW OF THE DIGESTIVE SYSTEM

1.1.1. Anatomy

The digestive system is constituted by the gastrointestinal tract, a hollow tube that extends from the mouth to the anus, and its accessory organs, primary glands that secrete fluids into the tract (Graaff, 2001).

The GI tract can be divided in several regions, each with its associated accessory organs (Table 1.1 and Figure 1.1).

Most of the digestive viscera are located within the abdominal cavity. Some of these viscera are covered by a serous membrane, composed of simple squamous epithelium and connective tissue, continuously secreting a serous fluid to lubricate the associated organs, providing protection, support and structural passage for vessels and nerves. This membrane is called Peritoneum (Graaff, 2001).

The peritoneum has a portion that lines the abdominal cavity's wall, the parietal peritoneum, and another that covers the internal organs, the visceral peritoneum. Two layers of parietal peritoneum come together in the posterior abdominal cavity to form a fold, the mesentery, supporting the GI tract and allowing passage of the nerves and vessels. The kidneys, the adrenal glands, the abdominal aorta, the urinary bladder, most of the pancreas, the duodenum, the ascending and descending colon, as well as the rectum, are located behind the parietal peritoneum, lying against the abdominal wall, being designated as retroperitoneal (Graaff, 2001).

Table 1.1 - Digestive system: GI tract and associated organs. Adapted from Graaff, 2001; Seeley et al., 2004; Standring, 2011

GI tract regions		Functions	Accessory organs
Oral cavity		Ingestion; mastication; digestion; deglutition.	Salivary glands
Pharynx		Deglutition.	
Esophagus		Propulsion.	
Stomach		Storage; digestion; absorption; mixing and propulsion.	
Small Intestine	**Duodenum** **Jejunum** **Ileum**	Neutralization; digestion; absorption; mixing and propulsion; excretion.	Liver Gallbladder Pancreas
Large Intestine	**Cecum** **Colon** **Rectum** **Anus**	Absorption (water and electrolytes); formation, storage and expulsion of feces.	

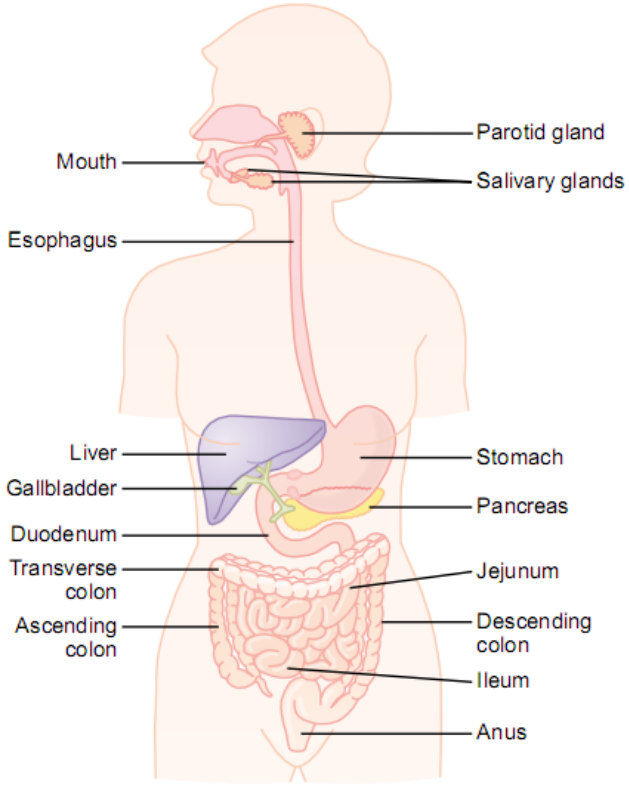

Figure 1.1 - Organs of the digestive system (Guyton, 2005).

1.1.1.1. Large Intestine

The large intestine can be subdivided in several regions: cecum, colon, rectum and anal canal (Figure 1.2). Being about 1.5 meters long, it comprehends about one fifth of the whole intestinal

length and extends from the end of the small intestine, the ileum, to the anus. Its aspect differs markedly from the small intestine, with a larger diameter, a sacculated shape forming the *haustra*, and the appendices *epiploicae*. The organization of the muscle fibers is also different, forming three longitudinal bands, the *taeniae coli*, that converge on the basis of the vermiform appendix and run along the entire length of the cecum and colon, being absent from the vermiform appendix, distal sigmoid colon and rectum.

The large intestine's course starts with a dilation, the cecum, in the right iliac fossa, ascends through the right flank (ascending colon), to the right hypochondriac region, where it bends, forming the right colic, or hepatic, flexure and crosses the mid upper abdomen (transverse colon) to the left hypochondriac region, bending downwards in the left colic, or splenic, flexure and descending through the left flank (descending colon) to the left iliac fossa where it forms the sigmoid flexure and runs posteroinferiorly into the pelvis (sigmoid colon), forming the rectum and ending in the anal canal. The anus is the external opening of the anal canal and is guarded by two sphincters: an internal, composed of smooth muscle, and an external, composed of skeletal muscle. Arising from the posteromedial wall of the cecum, exists a narrow, vermian tube designated vermiform appendix. It contains lymphoid tissue, which tends to decrease towards adulthood, along with the size of the appendix itself (Figure 1.2) (Standring, 2011) (Figure 1.2).

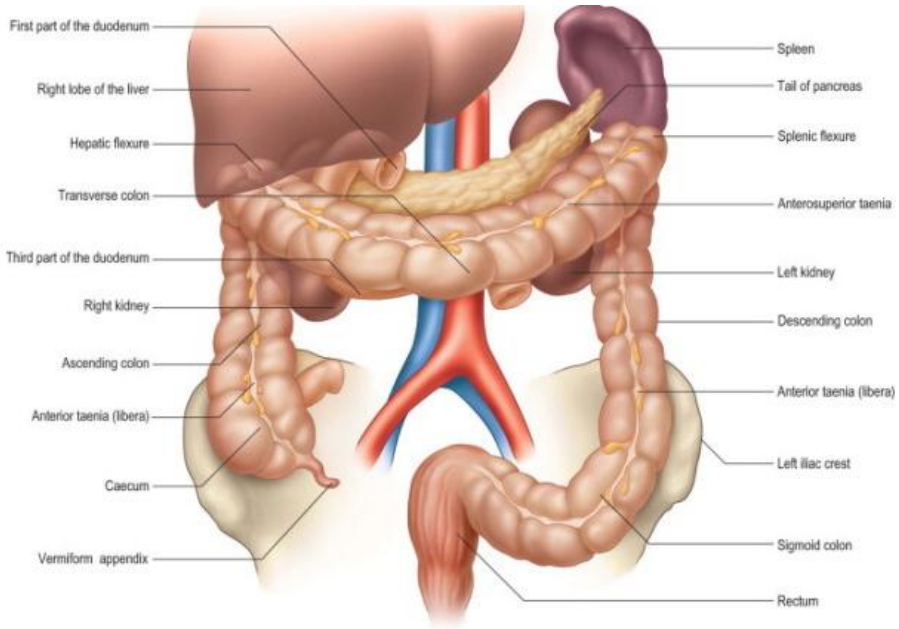

Figure 1.2 - Overview of the colon (Standring, 2011).

A careful comprehension of the vascular supply and lymphatic drainage systems are very important to understand the metastization pathways since these are two great conduits that can carry the disease to distant organs. Their structure and involvement in the disease must be therefore described in some detail (Libutti *et al.*, 2008).

Vascular Supply

The large intestine is supplied by branches of the superior and inferior mesenteric arteries through an arching arterial system (Guyton *et al.*, 2005) and drained mainly through the superior and inferior mesenteric veins to the portal vein (Figure 1.3 and Figure 1.4) (Standring, 2011).

The cecum is supplied by the ileocolic artery, the last right side branch of the superior mesenteric artery, which divides in superior and inferior branches and is drained by the corresponding veins to the superior mesenteric vein (Standring, 2011).

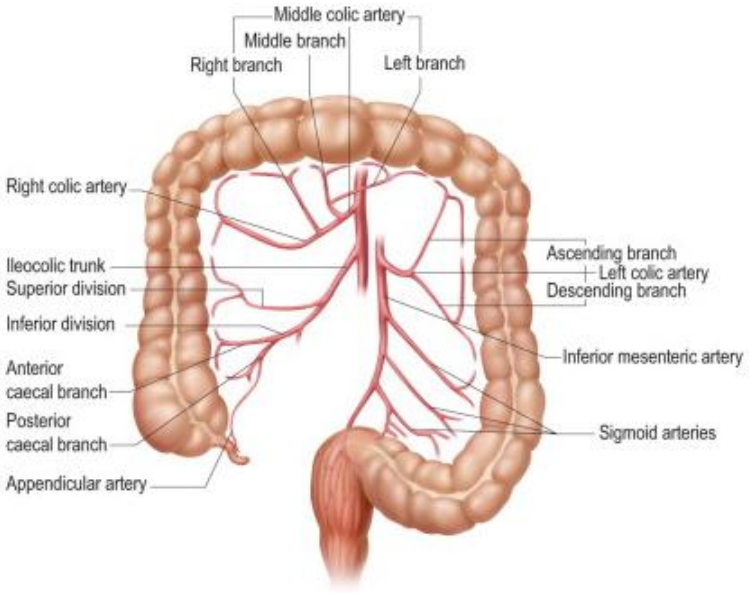

Figure 1.3 - Vascular supply of the large intestine (Standring, 2011).

The lower half of the ascending colon receives blood from the ascending branches of the ileocolic artery, which anastomoses with the descending branch of the right colic artery. The remaining ascending colon and part of the hepatic flexure are supplied by the ascending branch of the right colic artery, branch of the superior mesenteric artery, which anastomoses with the right branch of the middle colic artery. This section is drained by the corresponding veins to the superior mesenteric vein (Standring, 2011).

The proximal two thirds of the transverse colon are supplied by the middle colic artery, branch of the superior mesenteric artery. The distal third is usually supplied by the ascending branch of the left colic artery through the marginal artery, anastomosing with the left branch of the middle colic artery. The venous drainage is performed by several tributaries to the middle colic veins, which either drain to the superior mesenteric vein or directly to the portal vein (Standring, 2011).
The blood supply of the descending colon is provided by the left colic artery, branch of the inferior mesenteric artery. Its ascending branch supplies the splenic flexure and the descending branch provides blood to the remaining descending colon and anastomoses with the sigmoid arteries. This section is drained by the corresponding veins to the inferior mesenteric vein (Standring, 2011).

The arterial supply of the sigmoid colon is delivered by the sigmoid arteries, branches of the inferior mesenteric arteries. They also supply the lower descending colon, anastomosing with the descending branch of the left colic artery (Standring, 2011).

The upper two-thirds of the rectum are supplied by the superior rectal artery, continuation of the inferior mesenteric artery, which anastomoses with the sigmoid arteries. The middle third has some contribution from the middle rectal artery, branch of the internal iliac artery, when present. The distal third is supplied by the ascending branches of the inferior rectal arteries, terminal branches of the internal pudendal arteries. The vascular drainage of the rectum is carried out by the rectal venous plexus. It has an internal and an external part. The internal drains mainly to the superior rectal vein, the start of the inferior mesenteric vein, but connects widely with the external plexus. The superior portion of the external also drains to the superior rectal vein, while the middle portion is drained by the middle rectal vein and the inferior to the inferior rectal vein, into the internal pudendal vein (Standring, 2011).

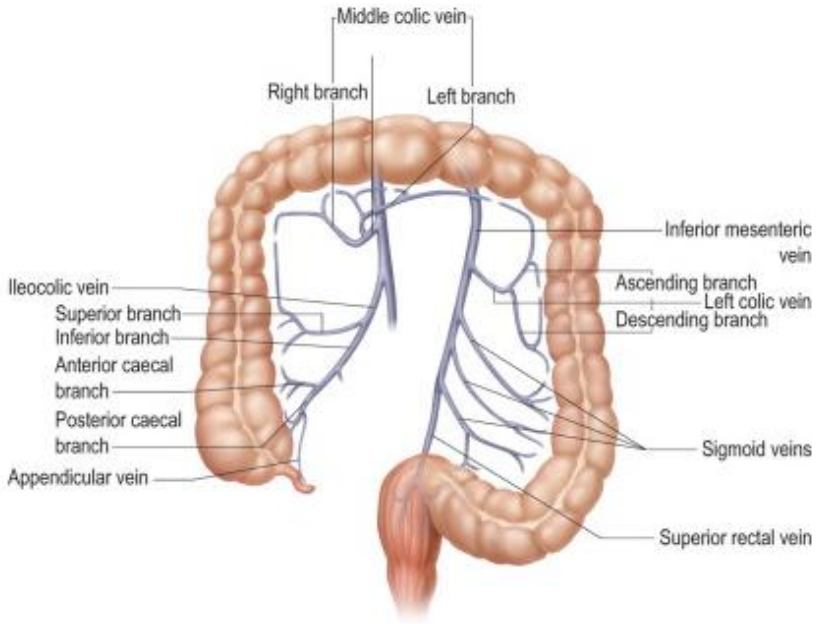

Figure 1.4 - Venous drainage of the large intestine (Standring, 2011).

The blood supply of the anal canal is derived from several arteries: superior rectal artery, inferior rectal branch of the pudendal artery and branches of median sacral artery. The venous drainage of the upper anal canal is done by the terminal branches of the superior rectal vein into the inferior mesenteric vein. The lower anal canal and sphincter are drained through the inferior rectal branch of the pudendal vein into the internal iliac vein (Standring, 2011).

Lymphatic drainage

The lymphatic vessels of the large intestine drain to nodes following the course of the main arteries that supply the colon – the superior and the inferior mesenteric arteries (Figure 1.5) (Standring, 2011).

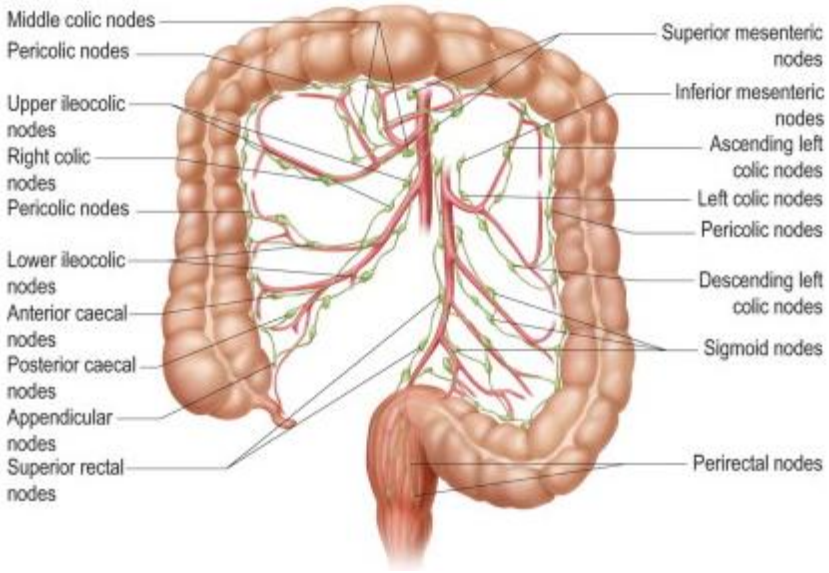

Figure 1.5 - Lymphatic drainage of the large intestine (Standring, 2011).

The nodes can be divided into four groups, according to their location: epicolic, the ones closest to the intestine, which lie on the serosa, sometimes in the appendices *epiploicae*; paracolic, located on the borders of the colon; intermediate colic, positioned along the colic vessels (ileocolic, right, middle and left colic, sigmoid and superior rectal arteries) and preterminal colic, which lie alongside the main trunks of the superior and inferior mesenteric arteries. These last nodes drain into para-aortic nodes at the source of those vessels (Standring, 2011).

The lymphatic drainage of the anal canal is divided between the lymphatics of the rectum (upper anal mucosa, internal sphincter and conjoint longitudinal coat) and external inguinal lymph nodes (lower anal canal and external anal sphincter) (Standring, 2011).

The knowledge of the lymphatic drainage is of crucial importance to understand the metastatic potential of colorectal cancer and influences surgical decisions, based on the location and local tissue spread of the tumor (Standring, 2011).

1.1.2. Histology

1.1.2.1. Gastrointestinal tract

The histology of the GI tract has similar traits throughout its different segments. The tract wall is formed by four distinct layers: mucosa, submucosa, muscularis externa and serosa (Figure 1.6) (Junqueira *et al.*, 2004;Standring, 2011).

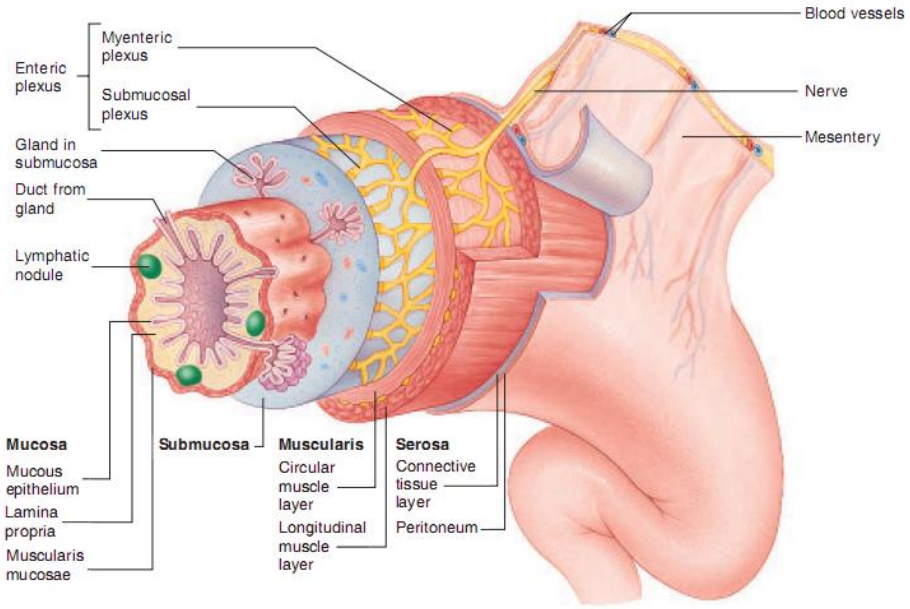

Figure 1.6 - General histology of the GI tract (adapted from Seeley *et al.*, 2004).

The mucosa is constituted by epithelium, lamina propria and muscularis mucosae.

The submucosa is composed of connective tissue with blood and lymphatic vessels and the submucosal (Meissner) nervous plexus. It may also contain glands (restricted to the duodenum) and lymphoid tissue (Junqueira *et al.*, 2004;Standring, 2011).

The muscularis externa has external longitudinal and internal circular layers of smooth muscle cells. Between these two layers is located the myenteric (Auerbach) nervous plexus (Junqueira *et al.*, 2004;Standring, 2011).

The serosa is referred above as visceral peritoneum and is composed of a layer of simple squamous epithelium, covering connective tissue with blood and lymphatic vessels, as well as adipose tissue (Junqueira *et al.*, 2004;Standring, 2011).

1.1.2.2. Large intestine

Mucosa

Epithelium

The luminal surface of the large intestine is lined by several types of cells: columnar, mucous, microfold, stem and neuroendocrine. These cells are not all evenly distributed. While columnar and mucous cells are the most abundant and present through the whole length of the large intestine, microfold cells are restricted to the epithelium overlying lymphoid follicles and both stem and neuroendocrine cells are mainly located at the base of the intestinal crypts. The crypts, in the large intestine, are long, numerous and are located close together (Standring, 2011).

Lamina Propria

The lamina propria of the large intestine is composed of connective tissue. It contains solitary lymphoid follicles, most abundant in the cecum, appendix and rectum, from which originate efferent lymphatic vessels (Standring, 2011).

Muscularis Mucosae

The muscularis mucosae is composed by internal circular and external longitudinal layers of smooth muscle cells (Standring, 2011).

Submucosa

The submucosa of the large intestine is similar to that of the remaining GI tract described above (Standring, 2011).

Muscularis externa

The aggregation of the longitudinal muscle fibers in three bands forms the taeniae coli, leaving only a thin layer of longitudinal fibers between them. The circular fibers form a thick layer in the rectum and the internal anal sphincter in the anal canal; in the cecum and remaining colon, they form a thin layer, particularly aggregated in between the sacculations (Standring, 2011).

Serosa

In the large intestine, the visceral peritoneum forms small appendices filled with adipose tissue – appendices *epiploicae* (Junqueira *et al.*, 2004). These are most numerous in the sigmoid and transverse colon, but generally absent from the rectum (Standring, 2011).

1.2. COLORECTAL CANCER

Colorectal cancer (CRC) is one of the most common malignancies and has a high mortality rate. It is the third more frequently diagnosed neoplasm and the second leading cancer-caused death in the United States of America in both men and women (Edwards *et al.*, 2010). In Portugal, the statistics are slightly different. Here, cancer of the colon, rectum, rectosigmoid junction and anus is the leading cancer-caused death (Estatísticas da Saúde - 2005, 2006). Its death rate in Portugal (mainland) is 31.2 per 100000 inhabitants (Risco de Morrer em Portugal 2006 - Volume II, 2006). The European data from 1998 to 2002 indicates a death rate of 18.5 and 10.7 per 100000 inhabitants for man and woman respectively (Zavoral *et al.*, 2009).

The most important demographic factor in the etiology of CRC is age (Libutti *et al.*, 2008), but there are other contributing factors such as sedentary life style, diet(Baxter *et al.*, 2007) and possibly many others. However, identifying specific agents that influence the risk of developing such cancer is a great challenge (Libutti *et al.*, 2008).

Despite the improvements in early detection of CRC that allowed the decrease of the overall CRC mortality in the last decade, the incidence has risen in the population under fifty years of age, among both men and women (Edwards *et al.*, 2010).

Genetic predisposition has a strong influence in the risk of developing CRC. The involvement of a first-degree relative with CRC doubles the risk of harboring the disease and increases the likelihood to develop premalignant adenomas (Libutti *et al.*, 2008).

Familial diseases that lead to CRC are also a factor to take into account. Familial Adenomatous Polyposis accounts for 1% of all CRC incidence; 100% of the patients who harbor it will evolve into CRC if the colon is not completely removed (Libutti *et al.*, 2008). Patients with Hereditary Nonpolyposis Colorectal Cancer have an increased risk of 80% and an accelerated rate of progression to CRC. The disease accounts for 3% of all CRC. Hamartomatous Polyposis

Syndromes contribute to less than 1% of all CRC annually and affects mainly the pediatric and adolescent population (Libutti *et al.*, 2008).

1.2.1. Diagnosis

Colorectal cancer, when diagnosed early, has good curative probabilities, but the same is not applied to later detections. The overall five-year survival rate among CRC patients is 40-50% (Jemal *et al.*, 2006), but the five-year survival rates for localized, regional and distant stages of disease are 90, 70 and 12%, respectively (Edwards *et al.*, 2010). Unfortunately, the early diagnosis is not enough, since a high rate of patients diagnosed with early stage disease will develop a recurrence later.

1.2.1.1. Symptoms

Symptoms and other conditions, reflected in laboratory values, associated with CRC are shown in Table 1.2.

Table 1.2 - Symptoms and conditions associated with CRC. Adapted from DeVita, 2008.

Symptoms	
	Change in bowel habits;
	Weight loss;
	Weakness;
	Hepatomegaly;
	Jaundice.
	Lower GI bleeding;
	Abdominal pain;
	Change in appetite;
	Blood in stools (bright blood, melena or hemoccult positive stool, depending on the location and stage of the disease);
	Adenopathy;
	Obstructive symptoms.
Other conditions	
	Electrolyte derangements;
	Carcinoembryonic antigen elevation.
	Iron deficiency anemia;
	Liver function abnormalities;

The examination should include physical exam, patient and family history, laboratory tests, computed tomography and colonoscopy. The latter is considered the most sensitive method for screening (Libutti *et al.*, 2008) since it allows the visualization of the colon to find intestinal polyps

that may either harbor the disease or premalignant formations (adenomas) or find full-fledged carcinomas.

1.2.2. Tumor Markers

Tumor markers (TM) are endogenous substances that are found in blood, stools, tumor tissue or any other tissue, in an altered state, when a patient develops cancer or some benign conditions. They can be produced by the cancer cells themselves or by any other cell in the body in response to the disease.

There are different TM for different types of cancer. However, some of them may be altered in more than one type and in some benign conditions. Furthermore, some people do not present altered levels, especially in the early stages of the disease.

There currently are several biological markers identified for CRC. They can be arranged in three categories according to their biological substrate: serum, tissue and feces. Carcinoembryonic antigen (CEA), KRAS and Fecal Occult Blood Testing (FOBT) are, respectively, examples from these categories.

Carcinoembryonic antigen is a glycoprotein expressed in normal or neoplastic epithelia of the gut, liver, lung and breast, among other tissues. Belonging to the immunoglobulin supergene family, these molecules play a role in cell adhesion and cell surface recognition. Their expression is also correlated with differentiation and architecture of epithelia, colonization for microbial flora, signal transduction, interaction with cytoskeleton and they are also present in sweat glands (Metze *et al.*, 1996).

This marker was described for the first time in 1965 by Gold and Freedman (Gold *et al.*, 1965) and has been the target of many studies since then. Nowadays it is recommended as a tool for prognosis, postsurgical follow-up and monitoring therapy in advanced disease (Duffy *et al.*, 2007;Locker *et al.*, 2006).

Carbohydrate Antigen 19-9 (CA 19-9) (also a serum marker) is the second most investigated CRC serum marker and was described in 1979 by molecular hybridization techniques (Koprowski *et al.*, 1979). The use of CA 19-9 is not recommended either by the European Group on Tumor Markers (EGTM) (Duffy *et al.*, 2007), the American Society of Clinical Oncology (ASCO) (Locker *et al.*,

2006) or the National Comprehensive Cancer Network (NCCN) (Colon Cancer - NCCN, 2011;Rectal Cancer - NCCN 2011) for diagnosis, prognosis or surveillance. However, several authors report that it is useful as an indicator of poor prognosis and metastasis (Barillari *et al.*, 1992;Chen *et al.*, 2005;Yakabe *et al.*, 2010). Accordingly, the Japanese Society for Cancer of the Colon and Rectum (JSCCR) recommends its measurement in combination with CEA during postoperative surveillance (Yakabe *et al.*, 2010).

The response markers KRAS and/or BRAF genes are frequently mutated in CRC (Barault *et al.*, 2008). They are both used to identify patients that will not benefit from therapeutic agents that target the epidermal growth factor receptor (EGFR).

FOBT is a common test to detect blood in the stool (Ayham *et al.*, 2009). The problem with this marker is the fact that colorectal neoplasms are not the single pathological condition that releases blood to the colon/rectum and that bleeding occurs only sporadically in CRC (Ayham *et al.*, 2009).

The ideal tumor marker, apart from being obtained by minimally invasive procedures in any location and having an accessible cost, should rise when the smallest neoplastic lesion appears and increase only when a tumor is present. It should be produced by all neoplastic cells and its levels increase proportionally to tumor extent. Finally, it must be produced by all patients (Fernandes *et al.*, 2005).

Since this perfect biological marker does not exist, it is necessary to identify other parameters that, when used in combination, compose a more specific and sensitive tool to facilitate the diagnosis and the prognosis.

The extensive literature includes a vast number of parameters to manage colorectal cancer. The following sections describe the possible use of the ones found most useful or promising in the prediction of survival and/or recurrence.

1.3. PARAMETERS RELATED WITH SURVIVAL AND RECURRENCE

It is difficult to accurately stage malignancies before the surgical procedure. Additionally, patients with the same CRC cancer stage present different survival periods, making the identification of highly predictive prognostic factors of CRC of utmost importance. Several factors, related to the

evolution of the tumor and/or the general health status of the patients are possible candidates and should be investigated.

The following parameters and their relation with survival and/or recurrence are summarized in Figure 1.7 at the end of this section.

1.3.1. Clinicopathological parameters

The UICC - TNM classification divides the patients into groups, according to the invasiveness and spread of the disease. It is based in macro and microscopic evaluation of the tumor and imaging techniques. The continuous growth of knowledge provides information that further stratifies these groups and tailors the therapeutic options for patients with specific characteristics, having a great impact on clinical practice.

Of great interest is the accurate identification of the node-negative patients belonging to the high-risk group that will experience recurrence in five years' time (Benson *et al.*, 2004) and that may benefit from adjuvant therapy, similarly to node-positive patients. This decision has to be weighed individually for each patient and is considered in the most recent ESMO guidelines (Labianca *et al.*, 2010). These patients may present with one of the following: suboptimal regional lymph node sampling; poorly differentiated tumor; lymphovascular invasion; perineural invasion; occlusive or perforating tumor and pT4 stage (Labianca *et al.*, 2010).

The clinicopathological examination of tumor specimens' characteristics probably still has much information that can be used to that purpose. Some of these characteristics will be discussed in this section.

1.3.1.1. Perineural Invasion

Perineural invasion has been associated with a more aggressive phenotype in other pathologies, like head and neck cancer (Liebig *et al.*, 2009). Unfortunately, in CRC, its importance has not yet been fully established and it is frequently underreported (Liebig *et al.*, 2009). It has been established that a relation with Cancer-specific and Disease-free survival (DFS) exists in node-negative patients (Colon Cancer - NCCN, 2011). Therefore, PNI may provide useful information to distinguish stage II patients that may benefit from adjuvant therapy (Fujita *et al.*, 2007;Fujita *et al.*, 2003). In addition to node-negative patients, Fujita, *et al*. found that the presence of PNI was also statistically

related with DFS for stage III patients with colon cancer (Fujita *et al.*, 2007). However, its use may be more limited in node-positive patients than in node-negative.

Results relating PNI with poor tumor differentiation, higher stage and increased metastatic disease at time of diagnosis suggest a role in disease progression and in tumor metastasis that has yet to be clarified (Liebig *et al.*, 2009). In addition, it is possible that the presence of PNI influences local (Fujita *et al.*, 2007) and distant metastatic (Fujita *et al.*, 2007;Quah *et al.*, 2008) recurrences and may be used as a predictor for lymph node metastasis (Fujita *et al.*, 2007;Huh *et al.*, 2010).

In conclusion, PNI may offer much more information on staging, prognostic and therapy and it is relatively easy to evaluate in the routine pathological examination. Further studies are necessary to confirm this data.

1.3.1.2. Extra Nodal Tumor Deposits

Extra nodal tumor deposits are defined as *Irregular discrete tumor deposits in pericolic or perirectal fat from the leading edge of the tumor and showing no evidence of residual lymph node tissue, but within the lymphatic drainage of the primary carcinoma, are considered peritumoral deposits or satellite nodules and are not counted as lymph nodes replaced by tumor* (Colon Cancer - NCCN, 2011).

Puppa *et al.* (Puppa *et al.*, 2009) investigated three types of deposits that influence the patients' prognosis in CRC differently. Among them, they found that tumor deposits with irregular shape, infiltrative appearance, not surrounded by lymphocytes and typically in close association with large vessels or nerves had greater impact on the prognosis of stage III patients, taking them near the prognosis of stage IV patients. This kind of deposits had already been associated with greater impact on survival than lymph node metastases and other types of vascular invasion (Ueno *et al.*, 2007).

These deposits can be categorized in different ways, which results in different prognostic values (Ueno *et al.*, 2007). Nevertheless, they provide valuable information that cannot be disregarded during staging and that should be explored as much as possible.

1.3.1.3. Inadequate number of lymph nodes examined

The number of lymph nodes has been found to be a prognostic factor for CRC, with a minimum of 12 nodes to accurately identify stage II cancers (Colon Cancer - NCCN, 2011).

The removal of a large number of lymph nodes may have side-effects, such as lymphedema. An alternative could be the use of sentinel lymph node biopsy. However, its use in CRC is still experimental (Colon Cancer - NCCN, 2011) and there is a vast number of techniques used in breast cancer and melanoma that are not usable or, for the moment, fully adapted to CRC (Bembenek *et al.*, 2008).

1.3.1.4. Lymphovascular Invasion

Lymphovascular invasion is considered an independent prognostic factor regardless of cancer stage (Harris *et al.*, 2008). It has been associated with a greater risk of lymph node metastases (Huh *et al.*, 2010) and the involvement of extramural veins has been related with increased risk of liver metastases (Compton *et al.*, 2000).

Although LVI is a parameter with recognized value in the guidelines, there is a great deal of variability between the observers (Harris *et al.*, 2008), and while some do not find it significant as an independent factor (Fujita *et al.*, 2003), others show that it is significant in colon cancer, but not in rectal (Tsai *et al.*, 2009); Zlobec *et al* (Zlobec *et al.*, 2008) finds it significant together with Raf-1 Kinase Inhibitor Protein to distinguish stage II patients in the high-risk group. These results are in accordance with the works of Tsai (Tsai *et al.*, 2008) and Meguerditchian (Meguerditchian *et al.*, 2005).

Similarly to PNI, LVI is a parameter readily available in the specimen examination that should be reported more often and standards should be used in order to assess the correct strength of this factor and improve patient care.

1.3.1.5. Response markers

Genetic testing is very useful when it comes to predict response to some chemotherapeutic agents. Mutations of the KRAS gene are associated with shorter survival (Lièvre *et al.*, 2006) and decreased response to therapeutic agents that target EGFR. Moreover, these mutations are present

in about 40% of colorectal cancers (Barault *et al.*, 2008;Richman *et al.*, 2009). Mutation testing of codons 12 and 13 of exon 2 of KRAS gene is therefore recommended by the NCCN guidelines for colon and rectal cancer (Colon Cancer - NCCN, 2011;Rectal Cancer - NCCN 2011) before therapy is initiated in metastatic disease.

KRAS is not the only gene involved in the EGFR signaling pathway. BRAF is also an important marker to consider in anti-EGFR therapy, being mutated in <15% of colorectal cancers (Siena *et al.*, 2009). Although the mutation of BRAF is less frequent that the mutation of KRAS, the presence of wild-type (non-mutated) BRAF is thought to be of utmost importance for anti-EGFR therapy (Colon Cancer - NCCN, 2011;Di Nicolantonio *et al.*, 2008). Mutation testing of BRAF V600E allele is also recommended by the NCCN guidelines for colon and rectal cancer before therapy is initiated in metastatic disease.

1.3.2. Hematological and biochemical parameters

Several biochemical parameters have been evaluated in relation to CRC survival.

Dixon *et al*. analyzed alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, total bilirubin, prothrombin time, mean corpuscular volume, fibrinogen, hematocrit and creatinine. Univariate testing identified alkaline phosphatase, aspartate aminotransferase and total bilirubin as significantly associated with survival in patients with stage IV colon and rectal adenocarcinoma.

In another study, with patients with Dukes' A, B or C disease, abnormal liver function test values were not found to be statistically significant in univariate testing for survival (Wang *et al.*, 2000). In both studies, none of those parameters was found to be statistically significant for survival in multivariate analysis (Dixon *et al.*, 2003;Wang *et al.*, 2000).

Despite correlation of hemoglobin levels with survival has been found in some studies, no definite conclusions can be drawn as of yet. Low hemoglobin levels have been found to negatively influence survival in more than one study. Heys *et al*. found that relation in univariate analysis, although no statistically significant difference between the different Dukes' categories was found. In another study, it was found to be correlated with progression-free and overall survival, as well as distant relapse, but not with clinical response (Roldan *et al.*, 2010). Conversely, Gobbi *et al*. did not find it statistically significant for survival in any of the analyses.

1.3.2.1. Preoperative Serum Albumin

Cancer patients frequently present low serum albumin levels. These values are related with shorter overall survival in CRC, by univariate and/or multivariate analysis (Cengiz *et al.*, 2006;Dixon *et al.*, 2003;Heys *et al.*, 1998;Sun *et al.*, 2009) and tend to lower as the disease progresses. The decreased levels probably result from an ongoing systemic inflammatory response that translates in to a higher demand of aminoacids for acute-phase protein synthesis and immune and anti-oxidant defenses (McMillan *et al.*, 2001). The sources for these aminoacids are the serum proteins and the skeletal muscle (McMillan *et al.*, 2001). The loss of skeletal muscle will translate in changes in body mass and the nutritional deficit will eventually lead to the death of the patient. However, being a larger reservoir of protein than the serum, body mass decrease will be noticed much later than the changes in serum total proteins (Heys *et al.*, 1998) and, more specifically, in serum albumin values (McMillan *et al.*, 2001).

The importance of serum albumin concentrations on CRC management has not yet been fully clarified but by the revision of the aforementioned studies its decrease may present as an early sign of malignancy and should seize the clinicians' attention.

1.3.2.2. Serum Glucose

A characteristic related with hyperglycemia, hyperinsulinemia, has substantial information indicating that it may play an important role in CRC (Giovannucci, 1995;McKeown-Eyssen, 1994). The presence of insulin receptors has been observed in both normal and malignant colorectal cells (Wong *et al.*, 1985) and the latter have been shown to express insulin-like growth factors. (Cullen *et al.*, 1991) It is not, therefore, strange that the risen insulin values are related with colorectal malignancies. The great role that environmental factors like obesity and sedentary lifestyle, as well as others that promote insulin production and/or resistance, play in colorectal carcinogenesis is another hint that supports this position (Giovannucci, 2001).

In several studies, glycemic load (Franceschi *et al.*, 2001;Michaud *et al.*, 2005), glycemic index (Franceschi *et al.*, 2001) or sugar intake (Michaud *et al.*, 2005) have been found related with increased CRC risk. However, a meta-analysis by Mulholland *et al.* (Mulholland *et al.*, 2009) that included these studies and ten others, indicated that glycemic load and index intakes are not associated with risk of CRC.

Some investigators used blood glucose measurements to assess CRC risk. While Nilsen *et al.* (Nilsen *et al.*, 2001) found a positive association in woman and Schoen *et al.* (Schoen *et al.*, 1999) found it without discriminating for gender, Tsushima *et al.* (Tsushima *et al.*, 2005) did not (for either sex). On another study, Trevisan *et al.* (Trevisan *et al.*, 2001) revealed a relationship between high blood glucose and risk of death by CRC.

The study of colorectal adenomas, the stage that precedes CRC, has provided contrasting results. While most authors have found a positive relation between blood glucose and the formation of adenomas and one that this relation is even stronger between glucose and CRC, which may mean that glucose is involved in progression to CRC, Park *et al.* (Park *et al.*, 2000) found an inverse result.

1.3.2.3. Serum CEA Values

Carcinoembryonic antigen is the oldest tumor marker in use for CRC. In addition, it is the only parameter that gathers enough consensual information to allow a standardized use. Even though it was discovered in 1965, there is still much investigation concerning its use for CRC and some of its results will be discussed in this section.

CEA kinetic parameters

Kim *et al*.(Kim *et al.*, 2009) found that the CEA clearance via exponential kinetics is significantly related with better overall survival and disease-free survival while clearance via randomized kinetics is related with worse prognosis. The latter suggests failure of the complete resection of the tumor, the existence of micrometastasis or regrowth of the remnant tumors.

The doubling time (CEA-DT) and half-life time (CEA-HL) of CEA have been evaluated as predictors of prognosis or metastatic progression in CRC (Ito *et al.*, 2002).

Although the results of Ito *et al*. show significant correlation between preoperative CEA-DT and after-surgery survival, they have limited validity due to postoperative adjuvant chemotherapy and re-resection after development of relapse or metastasis. Nevertheless, CEA-DT has been found related with the outcome of patients with recurrent CRC in general,(Staab *et al.*, 1982) with liver metastasis (Koga *et al.*, 1999;Tanaka *et al.*, 2004) and capable of distinguishing between candidates for repeat hepatectomy and those patients at high risk of multiple early recurrences (Tanaka *et al.*, 2004).

The objective underlying the evaluation of postsurgical CEA-HL was to diagnose overlooked metastases. The results indicate that the prolongation of the half-life time might suggest the existence of metastases earlier and more rigorously than the increase of CEA level in the postoperative period after a prolonged regression. In addition, the evaluation of postoperative CEA regression gradient might be helpful to identify patients who are undergoing complete or partial response.

Preoperative CEA values

The need for identifying populations who benefit from regular postoperative CEA monitoring is related to the low percentage of patients that present elevated CEA levels prior to the recurrence (Park *et al.*, 2009).

Park *et al*. (Park *et al.*, 2009) found that only a reduced number of patients with normal preoperative CEA levels would present with elevated CEA levels before the diagnoses of a recurrence. Conversely, a greater percentage of the patients who presented with high CEA levels before the surgery, presented also with elevated levels before the diagnosis of the recurrence. This data corroborates the indication of the guideline of the American Society of Clinical Oncology (ASCO) (Locker *et al.*, 2006) of using preoperative CEA levels to assess its utility in the surgery follow-up phase.

The ASCO guideline (Locker *et al.*, 2006) recommends the use of preoperative CEA testing for assisting in staging and surgical treatment planning, providing prognostic information and aiding in assessing its utility for postoperative surveillance. In fact, preoperative CEA values have been recognized as a prognostic factor for CRC after curative surgery by several authors (Harrison *et al.*, 1997;Park *et al.*, 2009;Park *et al.*, 2005;Sun *et al.*, 2009;Takagawa *et al.*, 2008;Wang *et al.*, 2000). However, there is not a consensus about which value (cut-off) should be used to distinguish between higher-risk and lower-risk populations.

Whereas some authors use the standard cut-off value of 5 ng/mL (although the one described in the literature as the normal value of carcinoembryonic antigen in a healthy person is 2.5 ng/mL and 5.0 ng/mL in smokers (Dugdale, 2009a)) and based their investigations about preoperative CEA levels on that value (Filiz *et al.*, 2009;Wang *et al.*, 2000), others focused on finding the value that separates the different populations with greater accuracy. While Park *et al*. (Park *et al.*, 2005) found that patients who presented preoperative levels above 3.0 ng/mL, were at higher risk of developing a recurrence after the curative surgery, Takagawa *et al*. identified 10.0 ng/mL as the

optimal cut-off value. More investigation is necessary to establish a reference value to use in the clinic.

Patients with higher preoperative CEA values, present often with greater tumor diameter, lymphatic invasion, higher TNM stage and perineural invasion (Table 1.3).

These factors are measures of the development of the tumor, presumably, because a more advanced, larger and possibly metastasized tumor produces more CEA.

Table 1.3 - Clinicopathological factors related with high preoperative CEA values

Clinicopathological factor	References
Tumor diameter	(Takagawa *et al.*, 2008)
Lymphatic invasion	(Filiz *et al.*, 2009;Takagawa *et al.*, 2008)
UICC - TNM stage	(Filiz *et al.*, 2009;Park *et al.*, 2009;Park *et al.*, 2005;Takagawa *et al.*, 2008)
Perineural invasion	(Filiz *et al.*, 2009;Park *et al.*, 2009;Takagawa *et al.*, 2008)

On the other hand, several other characteristics have been described as not being associated with elevated preoperative CEA, like vascular invasion (Park *et al.*, 2009;Takagawa *et al.*, 2008), tumor location (colon/rectum) (Filiz *et al.*, 2009;Park *et al.*, 2009;Takagawa *et al.*, 2008) and histologic type (Filiz *et al.*, 2009;Park *et al.*, 2009;Park *et al.*, 2005;Takagawa *et al.*, 2008). The reason why vascular invasion, which also reflects development of the tumor, is not related with preoperative CEA levels is not clear. From the association established above between CEA values and the tumor characteristics, a clinician may be able to infer about the depth of invasion, tumor size and metastases.

Postoperative CEA values

Intensive postoperative follow-up routines, which include several of the following procedures - clinical assessment, blood tests, CEA levels, chest radiograph, fecal occult blood test, liver ultrasound, computerized tomography, colonoscopy, sigmoidoscopy, barium enema, and others - have been established as improvers of survival and/or predictors of recurrence (Renehan *et al.*, 2002;Rodriguez-Moranta *et al.*, 2006;Tjandra *et al.*, 2007;Wanebo *et al.*, 1978). These are valuable procedures because the intensive follow-up enables an earlier detection of recurrences, allowing more effective treatment or surgery.

The ASCO guidelines recommend postoperative CEA testing every three months for at least three years after diagnosis when the patient (with stage II or III disease) is a candidate for surgery or systemic therapy. If elevated CEA values are found and confirmed by retesting, further evaluation is performed.

Although postoperative CEA values have been recognized as predictors of recurrences (Filiz *et al.*, 2009;McCall *et al.*, 1994;Rodriguez-Moranta *et al.*, 2006;Tjandra *et al.*, 2007;Tsai *et al.*, 2009;Wanebo *et al.*, 1978;Watine *et al.*, 2001), these are not always preceded of a marked rise on CEA level. This happens for various reasons, and while some of them are probably yet to be discovered, others are already known such as the primitive tumor stage and location, as well as the location of the recurrence. The latter is frequently related with the location of the primitive tumor as will be discussed below.

When considering tumor stage, Hara *et al*. (Hara *et al.*, 2010) found that the elevation of CEA in patients with stage II disease, unlike in those with stage III, does not have a high probability of predicting recurrence due to its high false-positive rate. These results are in accordance with those obtained by Park *et al*. (Park *et al.*, 2006), who found no differences between high and low perioperative CEA values relatively to survival in patients with stage II CRC.

As far as the recurrence location is concerned, hepatic metastases and disseminated disease are the ones who elicit the greatest elevations in CEA values (Wanebo *et al.*, 1978). Local or pelvic recurrences frequently present normal or just slightly elevated CEA values (Wanebo *et al.*, 1978).

On the relationship between the recurrence location and the primitive tumor location, the liver is the most common location for recurrences of CRC (Hara *et al.*, 2010;Tsai *et al.*, 2009). Henceforth, if a patient presents with elevated CEA values, he has a strong possibility of harbouring a liver metastasis. On the other hand, local recurrences are more common in early relapses of rectal cancer (Tsai *et al.*, 2009) and, since local recurrences do not usually provoke brisk CEA changes, these may pass unnoticed if an intensive follow-up strategy is not applied (Rodriguez-Moranta *et al.*, 2006). The same follow-up strategy applies to patients with stage II tumors, who frequently develop undetected recurrences, but not to those with stage III. The study by Rodriguez-Moranta *et al*. found that patients with stage III cancer did not benefit from the intensive strategy in the first two years, presumably because CEA presents a much lower rate of false positive results in this cancer stage (Hara *et al.*, 2010)

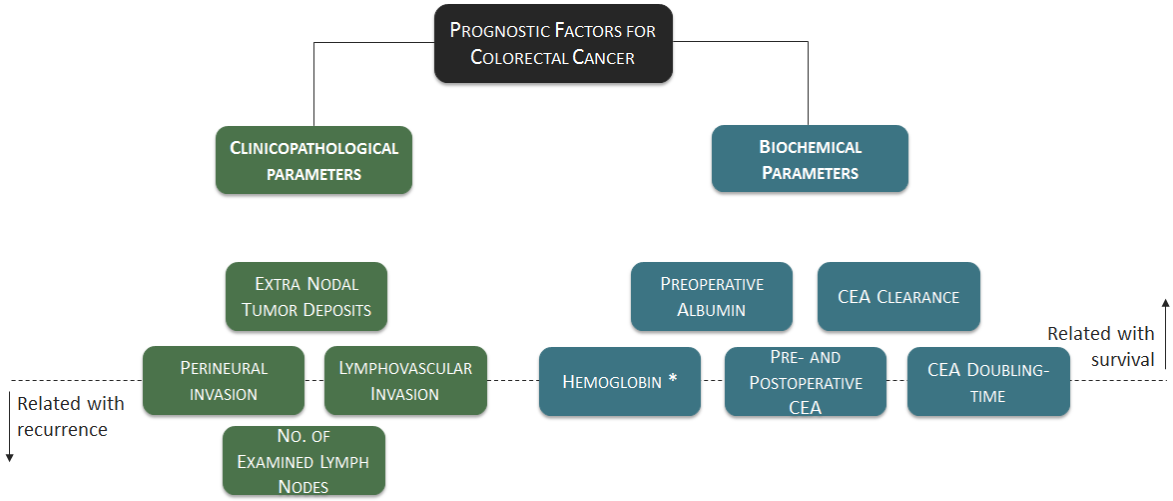

Figure 1.7 - Relations of CRC prognostic factors with tumor recurrence and patient survival.
Perineural Invasion (Colon Cancer - NCCN, 2011;Rectal Cancer - NCCN 2011); Extra Nodal Tumor Deposits (Colon Cancer - NCCN, 2011;Rectal Cancer - NCCN 2011); Lymphovascular Invasion (Fujita *et al.*, 2003;Tsai *et al.*, 2009) No. of Examined Lymph Nodes (Colon Cancer - NCCN, 2011); Hemoglobin (Heys *et al.*, 1998;Roldan *et al.*, 2010); Preoperative Albumin (Cengiz *et al.*, 2006;Dixon *et al.*, 2003;Heys *et al.*, 1998;Sun *et al.*, 2009); CEA Clearance (Kim *et al.*, 2009); CEA Doubling-time (Ito *et al.*, 2002;Koga *et al.*, 1999;Staab *et al.*, 1982;Tanaka *et al.*, 2004); Pre- and Postoperative CEA (Colon Cancer - NCCN, 2011;Locker *et al.*, 2006;Rectal Cancer - NCCN 2011).
*** There is still no consensus on the value of hemoglobin for CRC prognostic assessment (Section 1.3.2). It is included in this illustration because several studies point to some relation with survival and/or recurrence and it may yet be proven useful.**

CHAPTER 2: AIM

The aims of this dissertation are (a) to identify biochemical or clinicopathological parameters of patients with colorectal cancer, who have undergone curative surgery, that allow an earlier detection of recurrence, and (b) carry out a clinical investigation with non-standardized registries, while using retrospective data, gathered during the daily clinical practice.

To achieve it, we proposed to:

a) Gather retrospective patient data systematically;
b) Analyze the data with conventional statistical tools in univariant and multivariant methodologies.

CHAPTER 3: METHODS

3.1. ADMINISTRATIVE PROCESS

This project was submitted to the approval by the Infante D. Pedro E.P.E Hospital (IDPH) and University of Aveiro Joint Commission on the 28th of September of 2010. It was approved on the 15th of October of the same year and submitted to approval by the Administration of IDPH and the Ethics Committee of the same institution on the 19th of October of 2010. The approval was obtained on the 22nd of November of 2010.

3.2. DATA COLLECTION

From the period between 2005 and 2010, 442 electronic patient files were screened for inclusion in the study. The ones that appeared to fill the inclusion criteria were requested to the archive because the electronic files did not have all of the desired information.

Retrospective clinical information was gathered for 190 patients from 2005 to 2010 with diagnosis of colorectal cancer at the Oncology and Surgery Services of IDPH according to the following inclusion criteria:

- Histologically confirmed diagnosis of colorectal cancer;
- Candidate for curative surgery;
- No perioperative mortality.

Only 153 patients were included in our analysis due to the restriction of our exclusion criteria (Figure 3.1):

- Concomitant non-related cancer;
- Lack of biochemical or clinical data in the file;
- Lack of curative surgery;
- Lack of free-of-disease period;
- Wrong diagnosis.

The following data was systematically collected from both physical and electronic files of each patient to excel sheets:

- Biochemical data

  - Liver function tests;
  - Renal function tests;
  - Carcinoembryonic Antigen (CEA);
  - Carbohydrate antigen 19-9 (CA19.9);
  - Serum proteins;
  - Glucose;
  - Cholesterol and triglycerides;
  - Hemoglobin;
  - Hematocrit;
  - Complete blood count.

- Clinicopathological data
  - Tumor Grade;
  - pTNM staging;
  - Lymphatic invasion and embolization;
  - Vascular invasion and embolization;
  - Perineural invasion;
  - Venous invasion;

- Clinical data
  - Date of surgery;
  - Date of death;
  - Date and place of recurrence;
  - Chemotherapy dates and medication;
  - Clinical history.

- Demographic data
  - Age;
  - Sex.

Besides the 37 patients excluded by the criteria, 2 more were removed from some analyses along the study for lack of data. (Figure 3.1).

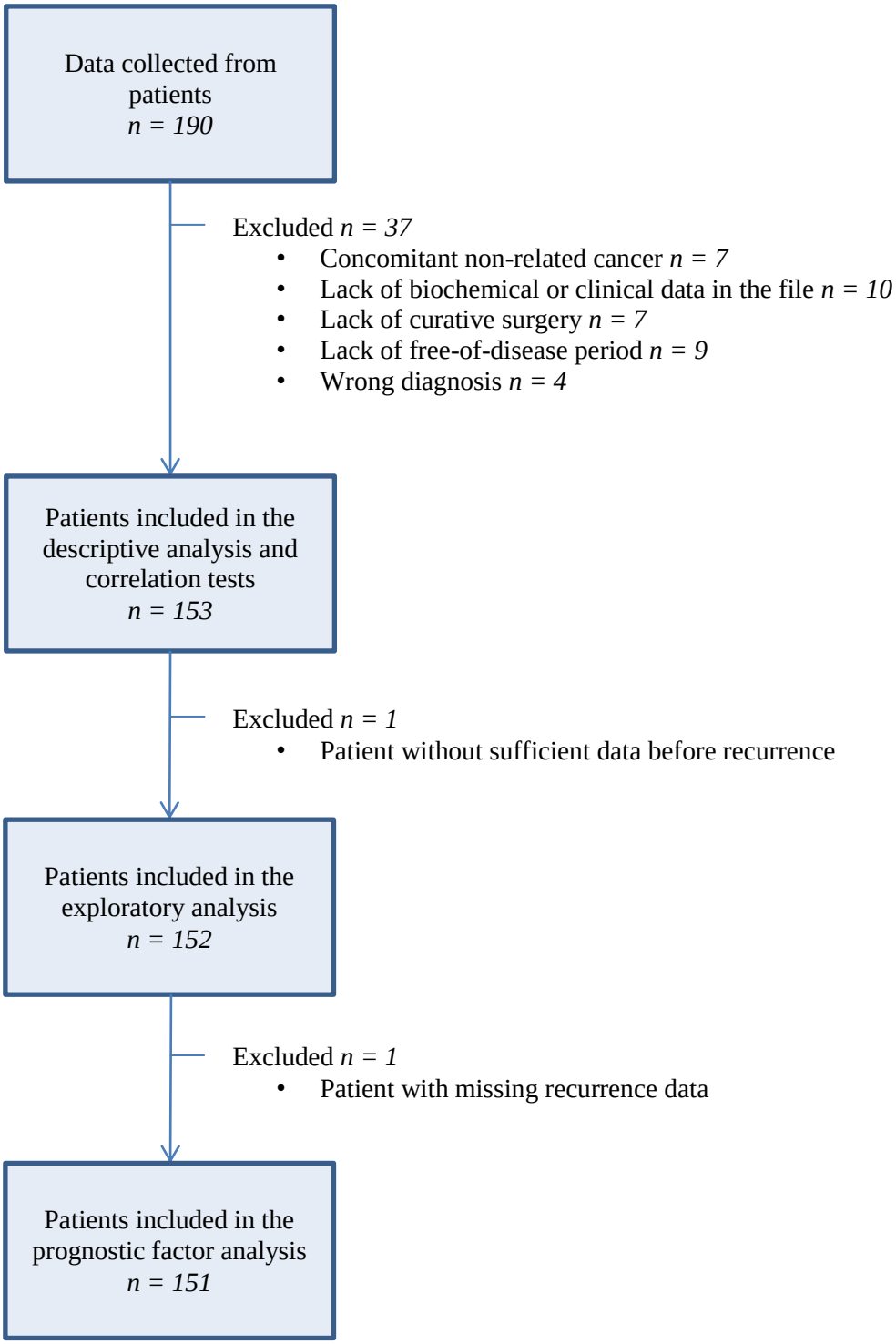

Figure 3.1 - Patient selection throughout the study.

The data was further separated into pre- and post-surgical periods. In the post-surgical period, 153 patients remained – being cut down to 151 throughout the study, while in the pre-surgical, only 107 were present in the end, when this segment was analyzed.

3.3. STATISTICS

Preliminary descriptive statistics were obtained for all variables. Since they revealed that most variables did not follow a normal distribution, Spearman Rank Correlation test was used to assess the correlation between all the biochemical parameters and the tumor markers CEA and CA 19-9.

The data used in this study comprised several measurements of the same parameters for each patient, with a variable number of measurements. These measurements were scattered along the entire follow-up of the patient with a very irregular distribution. Henceforth, in order to capture a value that reflected the mean value, while accounting for the different time lapses between the measurements, the area under the curve was calculated for each parameter, with a minimum of two measurements, using the following formula, in which A_1, A_2, A_3 and A_4 are the areas of the trapezoids that build the concentration curve (Figure 3.2):

$$AUC = \frac{A_1 + A_2 + A_3 + A_4 + A_{...}}{t_2 - t_1}$$

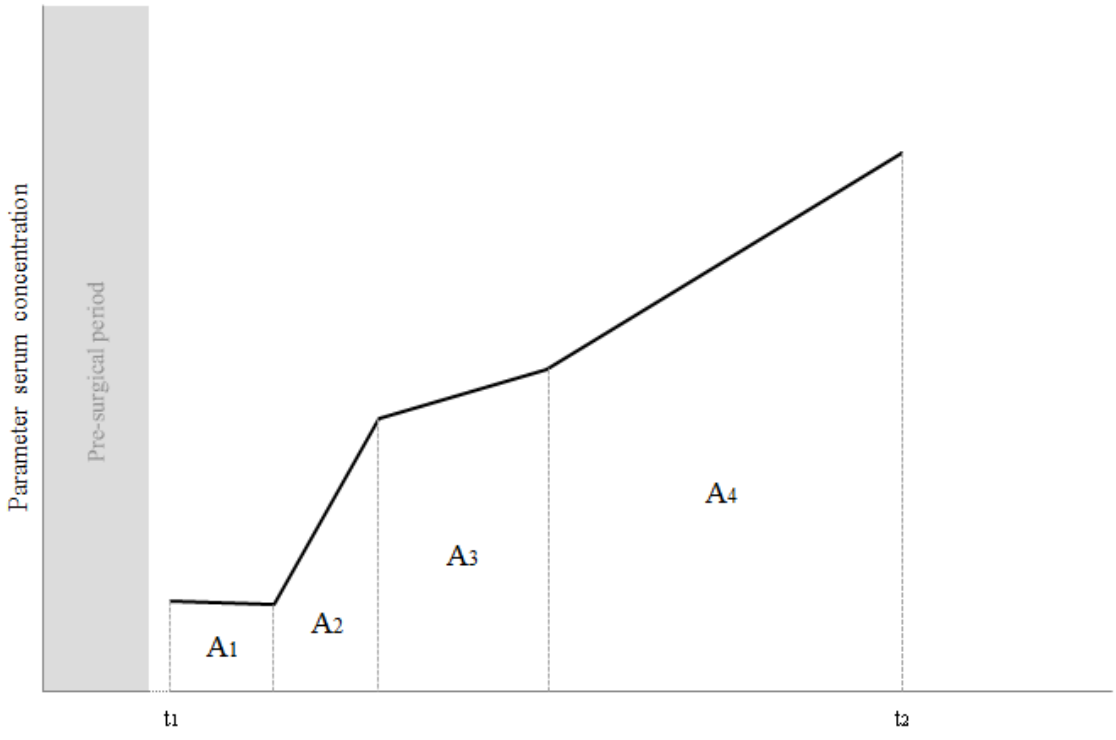

Figure 3.2 - Example of the calculation of the biochemical parameter's area under the curve. t1 is the concentration of the first measurement after the resective surgery and t2 is the last measurement of that parameter.

With the AUC calculated for all the parameters, an exploratory analysis of parameters related with recurrence was conducted.

Chi-square and Fischer exact test were used to analyze the distribution of the clinicopathological characteristics of the patients across subgroups of patients, with and without recurrence and the Mann-Whitney U test was used to compare the distribution of the biochemical parameters across the subgroups of patients according to recurrence status.

In order to determine the parameters that influenced cancer recurrence the most, three tools were used: The area under the Receiver Operating Characteristic (ROC) curves, to determine the parameters that best predicted the recurrence event and their cut-off with the best discrimination potential, Univariate Cox Proportional Hazards Model, to assess the risk of recurrence associated with each parameter and Multivariate Cox Proportional Hazards Model to evaluate the contribution of each parameter when analyzed in combination with others to recurrent disease prediction.

The pre-surgical data was the last to be analyzed. The same methods were used to test for prediction capability and associated risk.

The software used for the statistical analysis was IBM SPSS Statistics Base 17.0 for Windows® by IBM, New York, U.S.A. for most analyses, with the exception of the calculation of cut-offs, in which MATLAB® version 7.4.0 (R2007a) by MathWorks, Natick, Massachusetts, U.S.A. was used. Results with a *p-value* under 0.05 were considered significant.

3.4. STUDY DESIGN

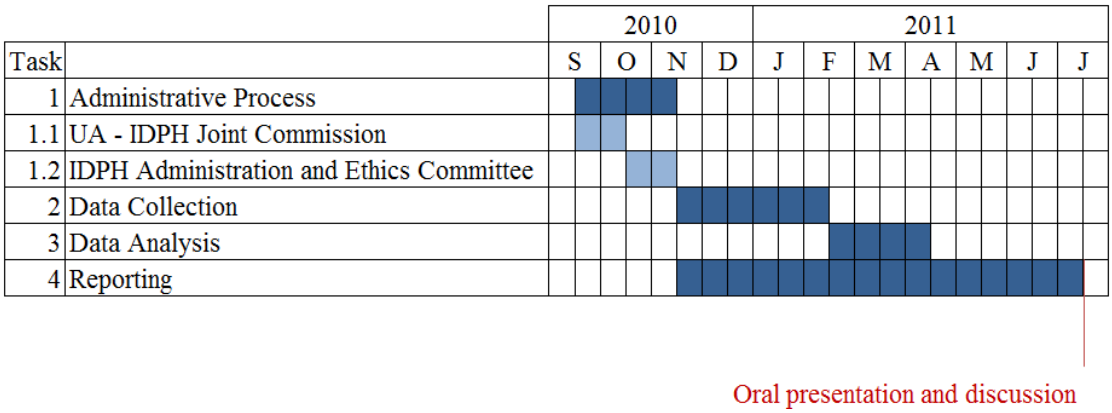

Figure 3.3 - Study design and schedule

CHAPTER 4: RESULTS

4.1. GENERAL DESCRIPTIVE STATISTICS

At the time of diagnosis, the mean age was found to be 67.95 (40 – 87; SE = 0.847) years with a mean time to recurrence of 21.12 (5.63 – 42.83; SE = 1.94) months. According to TNM Stage, six (3.9%) patients had stage 0 disease – stage with tumor *in situ*, twenty-four (15.7%) had stage I, fifty-eight (37.9%) had stage II, forty (26.1%) had stage III, six (3.9%) had stage IV and in nineteen patients (12.4%) this information was missing. Fifty-five patients (35.9%) had grade I tumors, fifty-eight (37.9%) had grade II, twenty-four (15.7%) had grade III, one (0.7%) had grade IV and this information was missing in 15 (9.8%) patients. By the end of the data retrieval period, twenty-eight patients (18.3%) had experienced recurrence, with only one (0.7%) patient lacking this information. The remaining one hundred and twenty-four patients (81%) did not experience such an event. Also, by the end of that same period, six patients (3.9%) had deceased and thirteen (8.5%) lacked that information. The remaining one hundred and thirty-four (87.6%) were still alive. Among those who had deceased, the mean survival time was 31.4 (19.3 – 36.6; SE = 2.77) months (Table 4.1).

Table 4.1 - Patient characteristics and clinicopathological parameters

Parameter	Patient count (%) *n*=153
Gender	
Feminine	69 (45.1)
Masculine	84 (54.9)
Patient Final State	
Alive	134 (87.6)
Deceased	6 (3.9)
Missing	13 (8.5)
TNM	
0	6 (3.9)
I	24 (15.7)
II	58 (37.9)
III	40 (26.1)
IV	6 (3.9)
Missing	19 (12.4)
Recurrence	
No	124 (81)
Yes	28 (18.3)
Missing	1 (0.7)
Recurrence Location	

Parameter	Patient count (%) *n*=153
Hepatic	16 (57.1)
Pulmonary	4 (14.3)
Local	5 (17.9)
Uterine	1 (3.6)
Peritoneal	1 (3.6)
Ovaric	1 (3.6)
Tumor Location	
Cecum	18 (11.8)
Ascending Colon	6 (3.9)
Hepatic Flexure	12 (7.8)
Transverse Colon	4 (2.6)
Splenic Flexure	5 (3.3)
Descending Colon	6 (3.9)
Sigmoid Colon	54 (35.3)
Rectosigmoid Junction	8 (5.2)
Rectum	38 (24.8)
Missing	2 (1.3)
Tumor Grade	
1	55 (35.9)
2	58 (37.9)
3	24 (15.7)
4	1 (0.7)
Missing	15 (9.8)
Number of Examined Nodes	
< 12	86 (56.2)
≥ 12	46 (30.1)
Missing	21 (13.7)
Vascular Embolization	
No	1 (0.7)
Yes	1 (0.7)
Missing	151 (98.7)
Vascular Invasion	
No	59 (38.6)
Yes	5 (3.3)
Missing	89 (58.2)
Lymphatic Embolization	
No	3 (2)
Yes	3 (2)
Missing	147 (96.1)
Lymphatic Invasion	
No	24 (15.7)
Yes	11 (7.2)

Parameter	Patient count (%) *n*=153
Missing	118 (77.1)
Venous Invasion	
No	7 (4.6)
Yes	9 (5.9)
Missing	137 (89.5)
Perineural Invasion	
No	13 (8.5)
Yes	8 (5.2)
Missing	132 (86.3)

The clinicopathological parameters that characterized the growth of the tumor into nearby vessels and nerves: vascular and lymphatic embolization and invasion and perineural invasion all have missing data over 50% and/or a very uneven distribution of data (Table 4.1).

From a total of 153 patients, there are 2995 entries in the biochemical database. The descriptive statistics of each parameter that is part of that database are presented in Table 4.2.

Table 4.2 - Descriptive statistics of biochemical data.

	n				
	Valid	**Missing**	**Median**	**Minimum**	**Maximum**
Erythrocytes	2879	116	4.2	1.8	6.5
Hemoglobin	2879	116	12.4	5.4	43.6
Hematocrit	2879	116	37.6	10.1	96.2
Mean Corpuscular Volume	2878	117	91.0	29.8	121.0
Mean Corpuscular Hemoglobin	2878	117	30.1	15.8	40.8
MCHC	2877	118	32.9	25.4	90.8
RDW	2812	183	13.7	9.9	31.5
Total White Blood Cells	2877	118	6.3	0.3	52.4
Neutrophils	2872	123	3.9	0.0	46.7
Eosinophils	2869	126	0.1	0.0	2.1
Basophils	2870	125	0.0	0.0	1.0
Lymphocytes	2871	124	1.5	0.0	11.1
Monocytes	2870	125	0.4	0.0	9.8
Platelets	2823	172	220.0	6.0	1291.0
Erythrocyte Sedimentation Rate	281	2714	20.0	2.0	128.0
Prothrombin Time	917	2078	12.1	9.6	53.6
International Normalized Ratio	915	2080	1.0	0.0	4.8
Thromboplastin Time	830	2165	28.2	12.4	155.0
Fibrinogen	30	2965	533.5	26.0	1149.0
D-Dimers	43	2952	362.0	76.0	2313.0
Glucose	2471	524	106.6	44.0	494.0
Glycated Hemoglobin	46	2949	6.9	4.4	63.1

	n				
	Valid	**Missing**	**Median**	**Minimum**	**Maximum**
Blood Urea Nitrogen	2434	561	36.0	0.8	261.7
Creatinine	2449	546	0.9	0.2	60.4
Sodium	2369	626	139.3	99.0	170.1
Potassium	2368	627	4.3	2.1	6.5
Chloride	2358	637	104.0	76.0	139.0
Calcium	793	2202	8.8	3.1	10.7
Phosphorus	371	2624	2.8	0.5	10.1
Magnesium	546	2449	2.0	1.0	4.2
Creatine Phosphokinase	203	2792	66.0	8.0	1997.0
Creatine Kinase-MB	89	2906	14.0	0.0	560.0
Troponin	79	2916	0.2	0.0	49.0
Myoglobin	59	2936	43.0	10.0	728.0
Aspartate Aminotransferase	1866	1129	21.0	6.0	1042.0
Alanine Aminotransferase	1843	1152	28.0	3.0	827.0
Lactate Dehydrogenase	1503	1492	191.0	32.0	2799.0
Bilirubin – Total	1385	1610	0.6	0.1	10.0
Bilirubin – Direct	1260	1735	0.1	0.0	5.7
Bilirubin – Indirect	1259	1736	0.5	0.0	4.3
Alkaline Phosphatase	1545	1450	89.0	25.0	2037.0
Gamma-Glutamyltransferase	1330	1665	35.0	4.0	1336.0
C-Reactive Protein	632	2363	2.7	0.0	44.0
Total Proteins	532	2463	7.0	2.7	8.6
Albumin	597	2398	3.8	0.7	5.2
Uric Acid	325	2670	5.0	0.0	10.3
Cholesterol – Total	261	2734	198.0	7.0	371.0
Cholesterol – HDL	227	2768	47.0	6.9	271.0
Cholesterol – LDL	195	2800	127.0	8.0	253.0
Triglycerides	239	2756	111.0	32.0	527.0
Amylase	182	2813	49.5	8.0	1123.0
Lipase	176	2819	53.0	1.0	4240.0
Iron	159	2836	70.8	8.0	361.0
Transferrin	79	2916	228.0	40.0	711.0
Transferrin Saturation	59	2936	21.4	0.2	125.4
Ferritin	125	2870	50.0	4.0	1253.0
Vitamin B-12	15	2980	390.0	277.0	751.0
Folic Acid	16	2979	9.3	3.3	33.4
Alpha-Fetoprotein	185	2810	1.9	0.9	20.5
CA 15-3	35	2960	26.0	7.0	1141.2
CEA	1139	1856	1.4	0.0	823.0
CA 125	61	2934	5.5	0.5	163.0
CA 19-9	1092	1903	10.8	1.2	462.9
PSA	127	2868	1.0	0.1	4172.0
PSA – Free	29	2966	0.2	0.1	11.9
T3	22	2973	21.3	1.3	264.0

	n				
	Valid	**Missing**	**Median**	**Minimum**	**Maximum**
T3 – Free	52	2943	3.0	1.1	186.0
T4	21	2974	7.6	4.4	104.8
T4 – Free	56	2939	1.2	0.5	15.1
Thyroid-Stimulating Hormone	81	2914	1.7	0.0	82.8
Follicle-Stimulating Hormone	1	2994	51.4	51.4	51.4
Luteinizing Hormone	1	2994	25.7	25.7	25.7

The variables that presented a number of entries (*n*) under 100 were excluded from the following analyses since they individually represent under 5% of the total entries and have not been measured in most individuals. Those variables were Fibrinogen (*n* = 30), D-Dimers (*n* = 43), Glycated Hemoglobin (*n* = 46), Creatine Kinase-MB (*n* = 89), Troponin (*n* = 79), Myoglobin (*n* = 59), Transferrin (*n* = 79), Transferrin Saturation (*n* = 59), Vitamin B-12 (*n* = 15), Folic Acid (*n* = 16), CA 15-3 (*n* = 35), CA 125 (*n* = 61), PSA – Free (*n* = 29), T3 (*n* = 22), T3 – Free (*n* = 52), T4 (*n* = 21), T4 – Free (*n* = 56), Thyroid-Stimulating Hormone (*n* = 81), Follicle-Stimulating Hormone (*n* = 1) and Luteinizing Hormone (*n* = 1).

Most parameters do not follow a normal data distribution. Only Erythrocytes, Hemoglobin, Hematocrit, Fibrinogen, D-dimers, Uric Acid, Total Cholesterol, LDL - Cholesterol, Iron, Transferrin, Transferrin Saturation, Vitamin B-12, Folic Acid and T3 presented a *p-value* > 0,05 in the Kolmogorov-Smirnov Test. Henceforth, non-parametric methods were used to further analyze the data.

4.2. POST-SURGICAL DATA ANALYSIS

The descriptive statistics of this section of the data are presented in Appendix, Table I-1.

The first step of the exploratory analysis of the post-surgical data was the investigation of relations between the biochemical parameters and the two tumor markers CEA and CA 19-9.

Since most parameters do not follow a normal distribution, Spearman Rank Correlation Test was used. It revealed that, besides being correlated with each other, the values of Lactate Dehydrogenase, Gamma-GT, C-Reactive Protein, Total Cholesterol, LDL Cholesterol, Triglycerides and Ferritin are significantly correlated with the values of either one or both the tumor markers (Table 4.3). Complete correlation results can be found in Appendix I, Table I-2.

Table 4.3 - Correlation coefficient of biochemical variables with CEA and CA 19-9.

	Spearman Correlation Coefficient	
	CEA	**CA 19-9**
CEA	---	0.281
CA 19-9	0.281	---
Lactate Dehydrogenase	0.356	0.179
Gamma-GT	---	0.284
C-Reactive Protein	0.294	---
Cholesterol – Total	0.350	---
Cholesterol - LDL	0.407	---
Triglycerides	0.287	0.382
Ferritin	---	0.317

Only statistically significant results are presented

We can observe that most of the parameters that correlate with CEA and CA 19-9 variations are related with inflammatory response: ESR and C-Reactive Protein; and liver function: AST, ALT, LDH, ALP, Gamma-GT, Cholesterol and Triglycerides.

The biochemical variables' distributions were tested for differences between the groups of patients with and without recurrence. The results that gathered both statistical significance and physiological interest are shown in Table 4.4 and complete distribution statistics across recurrence groups can be found in Appendix I, Table I-3.

Table 4.4 - Mann-Whitney U tests of biochemical variables across sub-groups of patients with and without recurrence.

	Recurrence		*p-value*
	No	**Yes**	
Total White Blood Cells	124	28	0.022
Glucose	124	28	0.010
Aspartate Aminotransferase	110	28	0.010
Lactate Dehydrogenase	102	28	<0.001
Alkaline Phosphatase	105	28	0.014
Gamma-GT	99	26	0.048
C-Reactive Protein	51	19	0.031
Albumin	60	21	0.034
CEA	113	23	<0.001
CA 19-9	114	23	<0.001

Only statistically significant results are presented.

That same analysis between the groups of patients alive and deceased would have been of interest. However, the low number of deceased patients – six – hindered that possibility.

An analysis of the clinicopathological parameters against tumor recurrence was impeded by the low frequency in those items. Even when Fischer Exact Test was applied, the differences were not significant (Table 4.5).

Table 4.5 - Chi-square tests of clinicopathological parameters *versus* recurrence.

	p-value
Vascular Invasion	0.578
Lymphatic Invasion	0.580
Perineural Invasion	0.400
Venous Invasion	0.308

For Tumor Grade, TNM Stage and Tumor Location the percentage of cells with expected counts less than 5 were 37.5, 50 and 25% respectively. Therefore, the Chi-square test cannot be applied and no data can be obtained for those parameters.

The only clinicopathological parameter that presented a sufficiently high frequency to provide relevant results in both groups, after crossed with tumor recurrence, was the Number of Examined Nodes. However, the results do not reach statistical significance although they come close to it, indicating that, as suggested in the current guidelines (Colon Cancer - NCCN, 2011;Rectal Cancer - NCCN 2011), under-sampling of lymphatic nodes poses a risk for CRC patients (Table 4.6).

Table 4.6 - Relation of Number of Examined Nodes *with* Recurrence. (No., Number)

		Recurrence		**Total**	χ^2	***p-value***
		No	**Yes**			
No. of Examined Nodes	**< 12**	64	21	85	3,592	0,058
	≥ 12	41	5	46		
Total		105	26	131		

4.2.1. Prognostic factors for recurrence

For this analysis, the AUC of each parameter was recalculated, considering only the period between the surgery and the recurrence, for the patients who experienced it and between the surgery and the end of follow-up for those who did not.

The prognostic factor analysis can be divided in 4 steps:

1. Correlation analysis of parameters with recurrence;
2. ROC curve analysis of each of the parameters and determination of the ideal cut-offs;
3. Univariate Cox regressions;
4. Multivariate Cox regressions.

The main struggle in this analysis is related with the low number of events – the recurrences (n = 27) and, additionally, the even lower number of patients with both the desired parameters and recurrence (Table 4.7).

Table 4.7 - Number and percentage of patients with recurrence for each of the analyzed parameters.

	Recurrence	
	Yes	**Total**
CEA	15 (11.7%)	128
CA 19-9	15 (11.6%)	129
Total White Blood Cells	27 (17.9%)	151
Glucose	26 (17.3%)	150
Lactate Dehydrogenase	20 (16.4%)	122
C-Reactive Protein	10 (16.4%)	61
Albumin	11 (15.5%)	71
Aspartate Aminotransferase	22 (16.7%)	132
Alkaline Phosphatase	22 (17.3%)	127
Gamma-GT	14 (12.4%)	113

Correlations

Table 4.8 - Correlations of the selected biochemical parameters with the occurrence of cancer recurrence.

	Correlation Coefficient	*p-value*
CEA	0.360	<0.0001
CA 19-9	0.248	0.005
Total White Blood Cells	0.268	0.001
Glucose	0.182	0.026
Lactate Dehydrogenase	0.239	0.008
C-Reactive Protein	0.224	0.083
Albumin	0.105	0.382
Aspartate Aminotransferase	0.096	0.272
Alkaline Phosphatase	0.006	0.950
Gamma-GT	0.040	0.671

This first step (Table 4.8), shows that only CEA, CA 19-9, TWBC, Glucose and LDH are correlated with recurrence, by only discarding the very low coefficient values and the not statistically significant. It does not provide specific information on the utility of each parameter as a predictor of recurrence but hints which may be useful. The next step confirmed these results.

Receiver Operating Characteristic Curve Analysis

The area under the ROC curve was calculated to determine the best value (cut-off) to trigger an alert of recurrence for each parameter.

From the calculations of sensitivity, specificity and accuracy for several cut-offs, the predictive capability and best cut-off were found for each parameter. The ones that did not reveal statistical significance were excluded from the next step.

Table 4.9 - Area under the ROC curve, best cut-off, sensitivity, specificity and accuracy of the selected parameters for predicting cancer recurrence.

	ROC AUC	*p-value*	Cut-off	Sensitivity	Specificity	Accuracy
CEA	0.823	< 0.0001	3.4 ng/mL	0.533	0.929	0.883
CA 19-9	0.724	0.005	14.6 ng/mL	0.533	0.746	0.721
TWBC	0.702	0.001	$6.8x10^3/\mu L$	0.519	0.677	0.649
Glucose	0.639	0.026	119.5 mg/dL	0.500	0.726	0.687
LDH	0.686	0.009	205.8 U/L	0.500	0.686	0.687
CRP	0.675	0.080	---		---	---
Albumin	0.416	0.378	---		---	---
AST	0.575	0.270	---		---	---
ALP	0.504	0.949	---		---	---
Gamma-GT	0.465	0.669	---		---	---

Although the traditional approach is to find the value that makes the best balance of specificity and sensitivity, due to the low number of events available, we considered the best cut-off for each parameter as the one with best accuracy after 50% of the events (recurrence) had been detected. That explains the sensitivity near 0.5 of all the parameters' cut-offs in Table 4.9 since the value with greater accuracy after 50% of the recurrences had been detected was usually that same value.

On this analysis, five factors were identified with prognostic capability: CEA, CA 19-9, Total TWBC, Glucose and LDH (Table 4.9)

Gamma-GT predictive capability was also calculated only for hepatic recurrences, not reaching statistical significance with $p = 0.840$.

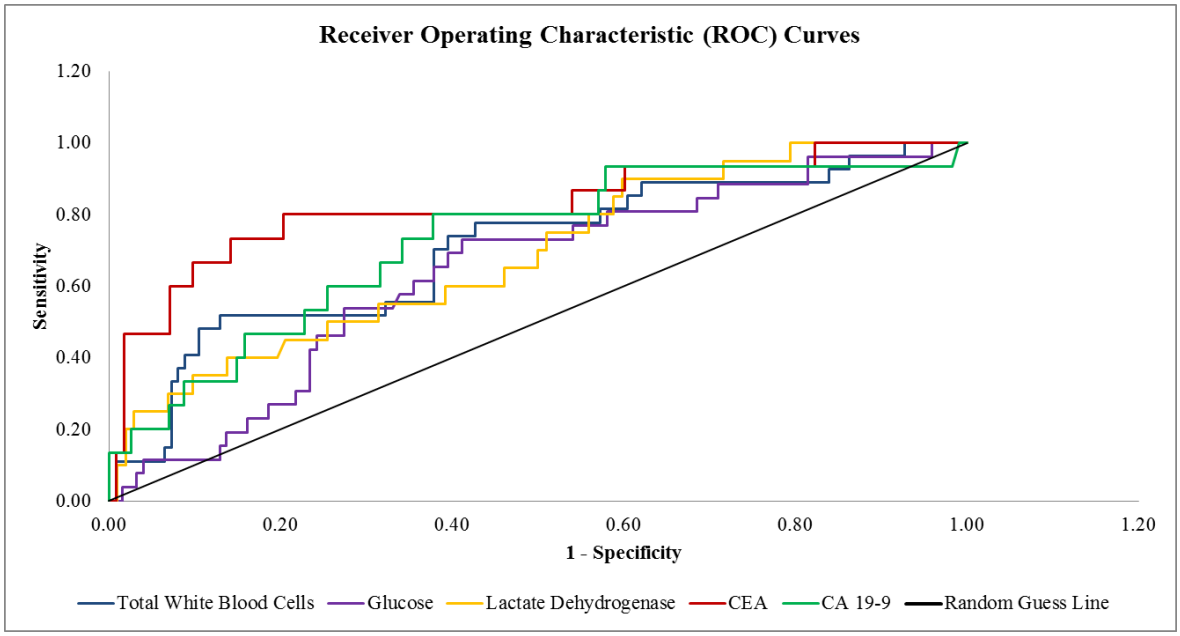

Figure 4.1 - ROC Curves of the selected parameters with predictive capability.

The curve of Carcinoembryonic antigen clearly stands out from the remaining parameters in Figure 4.1. The closest a curve gets to the top left corner, the better predictive properties that parameter possesses because it combines the highest specificity and sensibility.

Univariate Cox regressions

The individual predictive capability of each parameter was assessed with univariate Cox regressions.

Table 4.10 - Univariate analysis with Cox proportional hazards model of the parameters with predictive capability of tumor recurrence.

	p-value	Hazard Ratio (95% CI)
CEA (>3.4 ng/mL)	<0.0001	8.494 (3.096 – 23.506)
CA 19-9 (>14.6 ng/mL)	0.038	2.938 (1.064 – 8.112)
TWBC (>6.8x10^3/µL)	0.032	2.296 (1.075 – 4.907)
Glucose (>119.5 mg/dL)	0.021	2.478 (1.144 – 5.367)
Lactate Dehydrogenase (>205.8 U/L)	0.049	2.424 (1.004 – 5.852)

All parameters analyzed by the Cox proportional hazards model in this step provided significant results (Table 4.10). The difference between CEA values under and over 3.4 ng/mL is the most powerful predictor of recurrence in this analysis, followed by CA 19-9, although the latter is much closer to the remaining parameters. The remaining parameters appear to have similar prognostic properties.

Multivariate Cox regressions

After introducing all the factors with univariate $p < 0.05$ in a Cox proportional hazards model, Glucose and CEA were the only parameters identified as independent prognostic factors of CRC recurrence (Table 4.11) with additional risks of 3.990 and 5.198, respectively.

Table 4.11 - Multivariate analysis with Cox proportional hazards model of the prognostic factors for tumor recurrence.

	p-value	Hazard Ratio (HR)	95% CI
CEA (>3.4 ng/mL)	0.012	5.198	1.429 – 18.903
Glucose (>119.5 mg/dL)	0.029	3.990	1.149 – 13.857
Lactate Dehydrogenase (>205.8 U/L)	0.083	---	---
TWBC (>6.8x10^3/µL)	0.390	---	---
CA 19-9 (>14.5 ng/mL)	0.675	---	---

Since CEA was the strongest predictor in the analysis, it was removed to check for other parameters that might stand out when it was not present.

Table 4.12 - Multivariate analysis with Cox proportional hazards model of Glucose, TWBC, LDH and CA 19-9 as prognostic factors of tumor recurrence.

	p-value	HR (95% CI)
Glucose (>119.5 mg/dL)	0.012	4.967 (1.415 – 17.434)
TWBC (>6.8x10^3/µL)	0.533	---
Lactate Dehydrogenase (>205.8 U/L)	0.025	3.835 (1.186 – 12.404)
CA 19-9 (>14.5 ng/mL)	0.229	---

Glucose and LDH stood out as independent prognostic factors of colorectal cancer recurrence with additional risks of 4.967 and 3.835, respectively (Table 4.12).

CEA in Stage II and Stage III patients

Of the factors that influenced CEA values in the prediction of recurrences presented in Section 1.3.2.3, some were evaluated. The results regarding tumor stage are presented here. Other factors are presented in Section 5.2.1.

Only patients with Stage II or III were selected and a ROC curve analysis was performed for the CEA's predictive capability of recurrences

Table 4.13 - Predictive capability of CEA in the sub-populations of stage II and stage III patients.

	p-value	Area under the ROC curve	95% CI
Stage II	0.002	0.854	0.688 – 1.000
Stage III	0.203	---	---

CEA has predictive capability in stage II but it does not in stage III by the analysis of the ROC Curves (Table 4.13).

4.3. PRE-OPERATIVE DATA

The descriptive statistics of this section, with the AUC already calculated for each parameter, are presented in Appendix I, Table I-4.

After the analysis of the post-surgical, the pre-operative data was analyzed while looking for early prognostic signs given by the parameters that were tested in the post-surgical period. The ROC curves were plotted but unfortunately, all results lacked statistical significance, except for Alkaline Phosphatase.

The best cut-off calculated for that parameter was 97.6 U/L with specificity, sensitivity and accuracy of 0.811, 0.545 and 0.750, respectively and an area under the ROC Curve of 0.726.

That parameter was included in a Cox proportional hazards model but it did not reach significance (Table 4.14).

Table 4.14 - *p-value*s of the ROC curve and Cox proportional hazards analyses of the selected parameters

	ROC curve	Univariate Cox Regression
	p-value	**Cut-off used (*p-value*)**
CEA	0.909	
CA 19-9	0.673	
TWBC	0.892	
Lactate Dehydrogenase	0.204	
C-reactive Protein	0.148	
Albumin	0.150	
Aspartate Aminotransferase	0.221	
Alkaline Phosphatase	0.024	>97.6 U/L (0.820)
Gamma-GT	0.212	

These results are probably due to an even lower number of patients than in the post-surgical analysis and consequentially diminished quantity of data for each parameter (Table 4.15). The number of patients dropped to 107 and the number of recurrences to 17.

Table 4.15 - Number and percentage of patients with recurrence for each of the analyzed parameters.

	Recurrence	
	Yes	**Total**
CEA	2 (9%)	22
CA 19-9	2 (11.1%)	18
TWBC	17 (15.9%)	107
Lactate Dehydrogenase	10 (23.8%)	42
C-Reactive Protein	8 (19.5%)	41
Albumin	3 (12.5%)	24
Aspartate Aminotransferase	14 (19.4%)	72
Alkaline Phosphatase	11 (22.9%)	48
Gamma-GT	8 (19.0%)	42

CHAPTER 5: DISCUSSION

The human body works like a great machine, composed of immense small mechanisms that interact with each other at many different levels. Some of these are known by current science but many still elude scientists and are slowly unraveled, bit by bit. The organism's response to cancer still has many unknowns and will continue to be slowly comprehended.

The biochemical analysis of the human blood has long been used to detect disturbances in the homeostasis that physicians know to be related with certain diseases. The different parameters evaluated in those analyses have reference ranges that are considered normal and science tells us that when one or more parameters are off the range, something is wrong.

Maybe this principle of the reference ranges can be taken a bit further and, with a proper sample and adequate statistical methods, smaller changes in these parameters are detected as predictors of events long before the parameters get out of the reference range, clinical symptoms appear or perhaps some relationships between parameters and diseases are discovered.

Based on that idea that maybe smaller changes in some parameters can be found related with pathological events, this study proposed to identify parameters that could be used as prognostic factors for colorectal cancer recurrences among clinical, clinicopathological and biochemical data of patients of the Oncology and Surgery Services of the Infante D. Pedro E.P.E Hospital. The data was collected in a retrospective method from the daily registries of those services.

The study had some limitations. Infante D. Pedro E.P.E Hospital sends the records of deceased patients to an outsourced archive and their recovery has an associated cost that was not covered by the budget of this project. This resulted in a sample with more "alive" patients that what would be expected in an investigation on CRC which is clearly visible in the Patient Final State parameter (Table 4.1). Consequently, and since most patients who develop a recurrence have a reduced survival, the number of recurrences was also much smaller than what was expected and would be desirable for the purpose of the study. In addition, the laboratory data was not the same for every patient, existing missing data in almost all parameters. This limited the analyses even further as seen in Table 4.7.

The first complete analysis carried out in this work concerned all the parameters included in the study. Most of those parameters were sequentially ruled out along the way according to the results

of the several statistical tools employed, until only the ones of most interest remained for the last analysis.

The parameters that were included in the prognostic factor analysis were chosen with basis on two criteria: (a) the differences of the distribution of their values in the groups with and without recurrence (Table 4.4) and (b) plausibility of the relation with colorectal cancer.

Hematological parameters like Erythrocytes, Hematocrit and Hemoglobin were not included because of possible chemotherapy influence. Neutrophils and Monocytes were not included since they are a partial parameter of the more global TWBC count. LDL Cholesterol was also significantly related with the occurrence of recurrences but since there were only two patients that had LDL values and experienced recurrence, it was not considered. The enzyme Lipase is usually involved in conditions related with the Pancreas (Dugdale, 2009b). Since this is not an organ usually targeted by CRC, the parameter was not further analyzed.

5.1. CLINICOPATHOLOGICAL PARAMETERS

This study was not able to address the parameters, except for one, introduced in Section 1.3.1, due to the low frequencies observed in those parameters (Table 1.3 and Table 4.5). Most of these characteristics were not frequently reported in the clinicopathologic reports. Moreover, their evaluation varies meaningfully from professional to professional and depends on the conditions of the surgical piece. Other authors have addressed the underreporting issue in a systematic way and concluded that these characteristics may offer more information than they currently do.

The only parameter that had sufficient data to be analyzed was the Number of Examined Nodes. The results did not reach statistical significance but came very close to it ($p = 0.058$), agreeing with the literature data on colon cancer, that assigns an increased risk to patients who had less than 12 nodes examined, recognizing it as an independent prognostic factor (Colon Cancer - NCCN, 2011). Conversely, the data that concerns rectal cancer exclusively has not provided conclusive results yet, but mentioned a negative influence of neoadjuvant therapy on the retrieval of nodes.

5.2. POST-OPERATIVE DATA

5.2.1. CEA

The positive results obtained in the Cox proportional hazards model confirm a familiar scenario where Carcinoembryonic Antigen is concerned: CEA is the main biochemical marker in colorectal cancer. This is in line with current guideline recommendations(Colon Cancer - NCCN, 2011;Duffy *et al.*, 2007;Labianca *et al.*, 2010;Locker *et al.*, 2006;Rectal Cancer - NCCN 2011) and a myriad of studies (Filiz *et al.*, 2009;McCall *et al.*, 1994;Rodriguez-Moranta *et al.*, 2006;Tjandra *et al.*, 2007;Tsai *et al.*, 2009;Wanebo *et al.*, 1978;Watine *et al.*, 2001). Although CEA is regarded as the best biochemical marker, TNM staging is considered the best prognostic factor in CRC. But since our distribution of data prevented the analysis of its relation with recurrence on an earlier phase, it was not included in the Cox regression model.

The patients who experienced recurrence present significantly higher CEA values than the ones who remained disease-free (Table 4.4 and Figure 5.1). The univariate Cox regression provided a HR of around eight ($p < 0.0001$) for CEA much higher than any other parameter, which points to a major contribution to the risk of recurrence associated with values over 3.4 ng/mL. When analyzed in a multivariate model, the HR values drop to around five ($p = 0.012$), while a great rise in the *p-value* occurs. The great difference between the univariate and multivariate results can be explained by the missing data: when the Cox proportional hazards model is calculated, the number of cases is given by the patients who gather data on both the status and the independent variables. On the univariate analysis, the maximum number of cases is considered for each parameter. Once additional variables are added to the model, the number of cases is given by the intersection of all the parameters and the status variable. Consequently, it gets progressively smaller with each additional parameter.

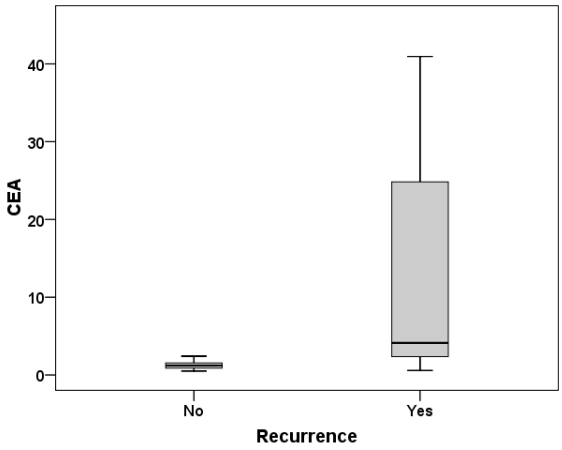

Figure 5.1 - Plot of the distribution of CEA values according to the ocurrence of CRC recurrence.

Together with the findings in the multivariate Cox proportional hazards model, which indicate that patients with serum CEA values above 3.4 ng/mL are between 1.429 and 18.903 times more likely

to develop a CRC recurrence than the others, with values below that level (Table 4.11), CEA is regarded as a good predictor of CRC recurrences.

The reference range of CEA is 5.0 ng/mL for all patients, except for smokers, which is 10.0 ng/mL, at the laboratory of the IDPH. Those are in agreement to the values described in the literature (Duffy *et al.*, 2003;Locker *et al.*, 2006), but there are some mentions of lower reference values, like 2.5 and 5.0 ng/mL for healthy people and smokers, respectively (Dugdale, 2009a).

In the present study, the optimal cut-off was found at 3.4 ng/mL. Although it is higher than the new value considered in the literature, it is within the reference range considered in the hospital's laboratory and recommended in the current guidelines from ASCO and EGTM that date from 2006 and 2007, respectively. In addition, no information was gathered on smoking habits of the patients, what may have skewed the analysis. The best cut-off was considered only after half the recurrences were detected, what may elicit a lower value than only finding the value with the best accuracy. Nevertheless, that value was chosen instead of the bibliographic reference because it was the one that reflected the characteristics of this patient sample.

Earlier in this text (Section 1.3.2.3), factors that interfere with the CEA pattern in the prediction of recurrences were referred and discussed: location and stage of the primary tumor, and recurrence location.

Regarding tumor stage, this study contrasts with the ones previously cited (Hara *et al.*, 2010;Park *et al.*, 2006). In fact, the results point to a greater predictive power in stage II patients than stage III, but one has to consider that by splitting the already meager recurrence sample, the results get progressively weaker (Table 4.13) and there are only eight patients with both CEA measurements and recurrence with stage II disease and five with stage III.

About recurrence location, the pattern of recurrence in this work does not allow a correct comparison since the group of liver metastases comprises more than half of the events and the remaining are scattered through other locations (Table 4.1). The data was further checked for a relationship between the initial place of the tumor and the place of the recurrence, but the relation between tumors of the rectum and local recurrences was not found (Table 5.1).

Reaching results that agree with the literature on CEA is a good indicator that the data, however incomplete, is able to produce reliable results.

Table 5.1 - Relation between the primary tumor's location and the location of the recurrences.

Recurrence Location	Primary Tumor Location	
	Colon	Rectum
Hepatic	10	6
Pulmonary	3	1
Local	4	1
Uterine	1	0
Peritoneal	1	0
Ovarian	1	0

5.2.2. CA 19-9

Carbohydrate Antigen 19-9 is not currently recommended in the European and American guidelines for diagnostic, prognostic or follow-up of CRC (Duffy *et al.*, 2007;Locker *et al.*, 2006). However, it is recommended by the Japanese Society for Cancer of the Colon and Rectum (Yakabe *et al.*, 2010) for postoperative surveillance along with CEA. Moreover, this marker is empirically used in the clinical practice and, being readily available, was included in the study.

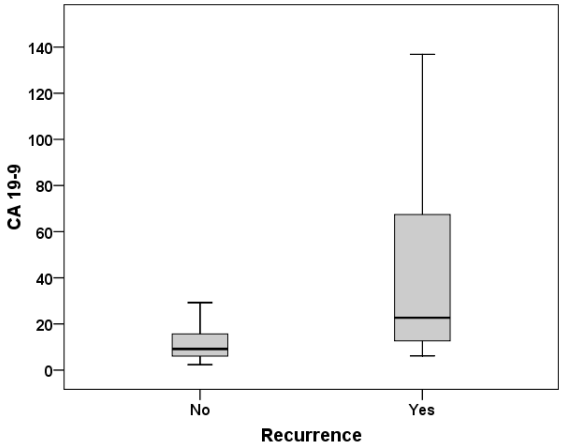

Figure 5.2 - Plot of the distribution of CA 19-9 values according to the ocurrence of CRC recurrence.

Unsurprisingly, it was not found to be an independent prognostic factor in the multivariate analysis, although it was significantly elevated in patients that developed recurrences (Table 4.4 and Figure 5.2) and patients with CA 19-9 above 14.6 ng/mL presented an increased risk of about three times (p = 0.038) for developing CRC recurrence by the univariate analysis (Table 4.10).

The results from the ROC Curve analysis and univariate Cox proportional hazards model show us that it has some predictive capability, but when the bigger picture is considered, in the multivariate analysis, CEA is a much more complete factor, predicting all the recurrences that CA 19-9 does, and more. Although unlikely, because of the entire bibliographic consensus on the lack of utility of CA 19-9, the inherent properties of the Cox proportional hazards model, explained in Section 5.2.1, may also contribute to the lack of significance of CA 19-9 in the multivariate model.

5.2.3. Glucose

Serum glucose was found to be a predictor of CRC recurrence in both uni- and multivariate analyses. Although no information was collected on dietary habits, height or weight, physical activity or lifestyle, high glucose levels may be related with some of these factors or an underlying disease like diabetes.

The contribution of glucose to CRC management is not clear. If, on one hand, some studies find in their results a positive correlation, on the other, when a broad analysis is made, the conclusions point otherwise (Mulholland *et al.*, 2009). The physiological rationale makes sense, but the attempts to bridge the glucose response or its levels with the effects of insulin on CRC and demonstrate those theories have not yet yielded conclusive results.

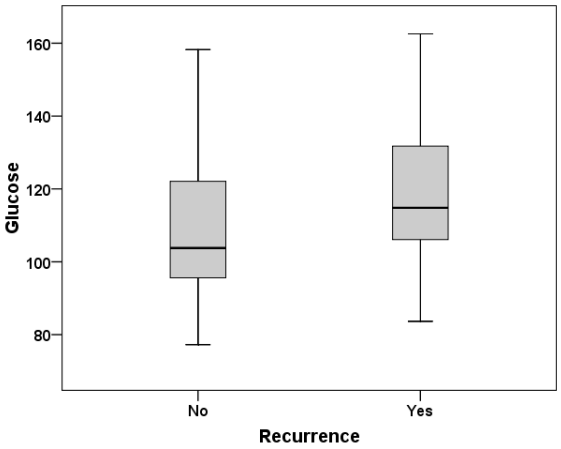

Figure 5.3 - Plot of the distribution of Glucose values according to the ocurrence of CRC recurrence.

To our knowledge, there is no study that assesses the risk of CRC recurrence with any of the aforementioned factors. In the current study, patients who develop recurrence have a slight, but significant (p = 0.010) tendency to higher blood glucose levels (Figure 5.3). The difference would probably be discarded if the parameter did not yield positive results in the following analyses (Table 4.9 to 4.12), indicating that that slight difference might be important. In fact, patients with blood glucose values over 119.5 mg/dL present a HR around 2.5, meaning that they have a risk of two and a half times greater of developing a recurrence than those patients with blood glucose values below that level. When a multivariate model is calculated, the HR associated with glucose actually rises to almost four in the patient sample (Table 4.11), contradicting the explanation provided in the Section 5.2.1 and maybe explaining part of the reduction in the risk associated only with CEA. The combination of these two parameters may contribute to an improved prediction of the recurrences.

This is also, to our knowledge, the first study to establish a cut-off for this parameter in the recurrence of colorectal neoplasms. Moreover, glucose was the only parameter that was found to be a prognostic factor in the multivariate analysis, along with CEA (Table 4.11). This either means that the selected cut-off does not discriminate as well as it should, although it is above the upper

limit of the reference range in the IDPH's laboratory, which is 60 – 110 mg/dL, or that glucose is in fact a good predictive factor for recurrence, at least for this patient sample.

The results are in line with the previous studies that related glucose values with adenoma formation and colorectal cancer.

Glucose must be taken into account with other characteristics of the patient like body measures, lifestyle, diet and other metabolic abnormalities. That data was not accounted for in this study and its collection would have been irregular due to the non-standardization of the information at the site. Nevertheless, these results are a hint of an approach to the use of this parameter in the big picture of colorectal cancer management.

The information on whether the glucose values were obtained in fasting or fed conditions was not available, what may limit our conclusions.

5.2.4. Liver Function Tests and Lactate Dehydrogenase

Parameters that are usually part of the liver function tests, Aspartate Aminotransferase, Alkaline Phosphatase and Gamma-GT, along with Lactate Dehydrogenase were selected in the inter-group analysis for that same reason: being related with liver function, since the liver is the main place of occurrence of distant metastases (Hara *et al.*, 2010;Tsai *et al.*, 2009).

Except for LDH, none of the other parameters demonstrated significant predictive capability in the ROC Curve analysis (Table 4.9) although they had proven to be significantly elevated in the patients who experienced recurrence (Figure 5. to Figure 5. and Table 4.4). There have not been many recent studies evaluating the part LFTs play in CRC management, but from a review of studies since 1974, there seems to be some uncertainty on the role of LFTs in the detection of CRC recurrences.

Rocklin *et al.* (Rocklin *et al.*, 1991) found that, when compared with CEA, the use of LFTs does not provide substantial additional information. Ohlsson *et al.* (Ohlsson *et al.*, 1995) examined intensive follow-up in CRC patients and besides the lack of evidence that the intensive follow-up provided additional benefit on a regular basis, within the blood tests, LFTs had a negligible contribution to the detection of recurrences, with CEA playing the main role. On yet another study, LFTs were found to be useful in the detection of metastases when CEA exceeded 500 ng/mL and

Gamma-GT 100 U/L. These are high values for these variables, which may render the measurement of LFTs useless because at such high CEA concentrations, imaging or surgical procedures would be triggered anyway (Cooper *et al.*, 1975).

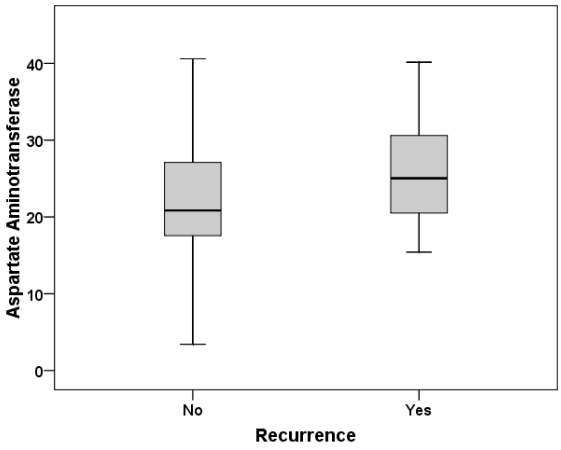

Figure 5.4 - Plot of the distribution of AST values according to the ocurrence of CRC recurrence.

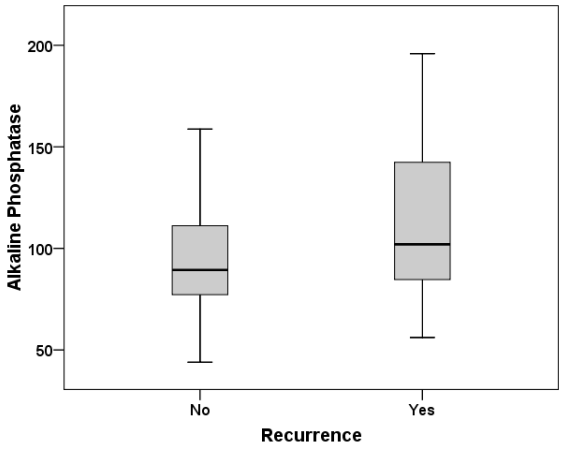

Figure 5.5 - Plot of the distribution of ALP according to the ocurrence of CRC recurrence.

Gamma-GT, when used in conjunction with CEA, has been found to help in the differentiation between hepatic metastases and others (Steele *et al.*, 1974). The low number of patients with hepatic recurrences and Gamma-GT (n = 8) measurements prevented a proper study of this characteristic in the present study, with the parameters not reaching statistical significance in the ROC Curve calculation.

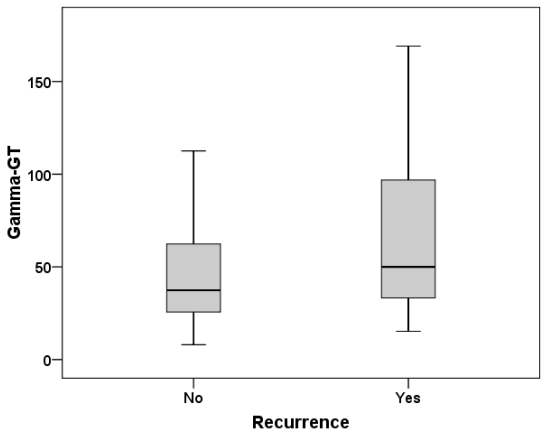

Figure 5.6 - Plot of the distribution of Gamma-GT values according to the ocurrence of CRC recurrence.

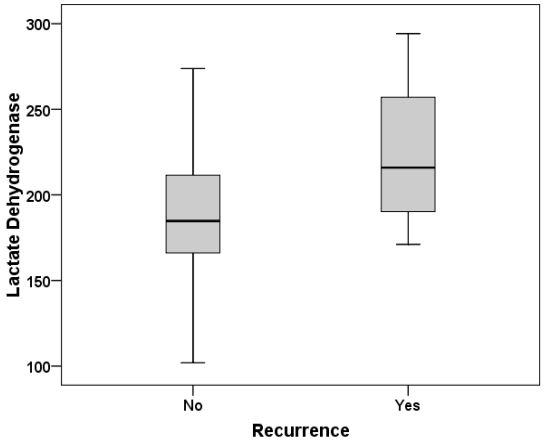

Figure 5.7 - Plot of the distribution of LDH values according to the ocurrence of CRC recurrence.

Lactate dehydrogenase does not specifically reflect liver function, although it is frequently requested along with the liver function tests. Instead, it reflects tissue breakdown (Vorvick, 2010). In case of colorectal cancer, it may reflect (a) the tissue damage caused by the tumor – e.g.: in the liver in the case of a metastasis or (b) the presence of tumor growth due to the high rate of cellular turnover. In either case, a rise in its values may raise a suspicion.

LDH took significantly higher values in patients who experienced recurrence (Figure 5. and Table 4.4). In addition, in the univariate Cox proportional hazards model, LDH values over 205.8 U/L presented an additional risk of about two and a half times greater than lower or equal values with a *p-value* of 0.049 (Table 4.10). Although these numbers are on the borderline of statistical significance, an analysis with a greater number of subjects would allow for a better determination of the real predictive power of this (and other) parameters.

When included in the multivariate analysis with the Cox proportional hazards model, LDH failed to contribute with any prognostic information that CEA did not already provide (Table 4.11). However, when CEA was removed from the analysis, LDH posed an additional risk of almost four times for the developing of recurrences (Table 4.12), alongside with Glucose.

Between the multivariate Cox regressions with and without CEA, there is no change in the number of cases. Henceforth, the differences observed in LDH are probably explained by the greater prognostic capability of CEA, whose rise poses a much greater risk for recurrence when compared with LDH.

5.2.5. Inflammation markers

The parameters of C-Reactive Protein, Albumin and TWBC were selected from the inter-group tests because they usually translate, to some degree, the body's response to an unknown entity or other stimulus in (a) an inflammatory response or (b) an infection. Since cancer cells are recognized by the immune system as foreign to the host, both these responses are frequently present.

5.2.5.1. CRP

C-Reactive Protein has been found useful in several studies (Aleksandrova *et al.*, 2010;Crozier *et al.*, 2006;Ishizuka *et al.*, 2009;Koike *et al.*, 2008;McMillan *et al.*, 1995;Sharma *et al.*, 2008). It has

been considered independently and together with albumin in an inflammation score named Glasgow Prognostic Score (GPS) which categorizes patients into three levels according to CRP and Albumin cut-offs of 10 mg/dL and 3.5 g/dL respectively. When CRP is elevated (CRP > 10 mg/dL) and hypoalbuminemia is present (Albumin < 3.5 g/dL) the score of 2 is attributed. When only one of those conditions is satisfied the score 1 is credited. Finally, when none is present, the allocated score is 0.

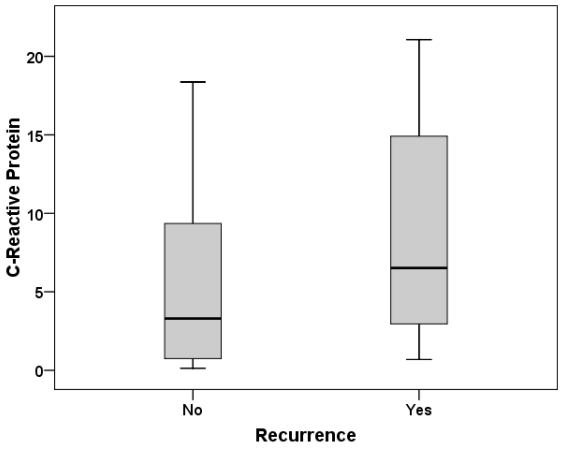

Figure 5.4 - Plot of the distribution of CRP according to the ocurrence of CRC recurrence.

In a brief summary of the studies mentioned above, CRP provided useful information related to (a) colon cancer (but not rectal) risk predominantly in men, (Aleksandrova *et al.*, 2010), (b) the prediction of survival (in the context of GPS) of patients with stage IV disease (Sharma *et al.*, 2008), (c) the need of adjuvant chemotherapy in stage II patients (preoperative CRP were used) (Koike *et al.*, 2008), (d) the prediction of poor outcome in patients receiving adjuvant chemotherapy (Crozier *et al.*, 2006), (e) patients with stage IV disease with liver metastases (Ishizuka *et al.*, 2009) and (f) the detection of recurrences (McMillan *et al.*, 1995).

The results of the present study show that there are more high CRP values in the group of patients that experienced recurrences with a $p = 0.031$ in Table 4.4 (Figure 5.4), but the ROC Curve analysis demonstrated that the parameter did not provide significant results towards the detection of recurrences ($p = 0.08$), maybe because there are also high values in the non-recurrence group, albeit in less quantity. Kwon *et al.* found similar results when evaluating preoperative CRP towards survival (Kwon *et al.*, 2010). The higher values in the recurrence group are consistent with studies that report elevated CRP in patients with advanced disease, but the absence of predictive capability is in contrast with the results obtained by McMillan *et al.* (McMillan *et al.*, 1995), since they found CRP to detect recurrences even earlier than CEA, even though they presented a small patient sample.

The patient sample in the present study may not have been large enough to grant statistical power to this parameter. However, it came close to statistical significance in the ROC Curve analysis, perceiving a possible improvement in a larger sample.

5.2.5.2. Albumin

As referred before, in Section 1.3.2.1, the decrease of preoperative serum Albumin may present as an early sign of malignancy. Accordingly, a systematic review by Gupta and Lis (Gupta *et al.*, 2010) found pretreatment albumin to be suitable for defining baseline risk in clinical trials in oncology, but also that there is a lack of knowledge on whether raised levels decrease the excess risk of mortality.

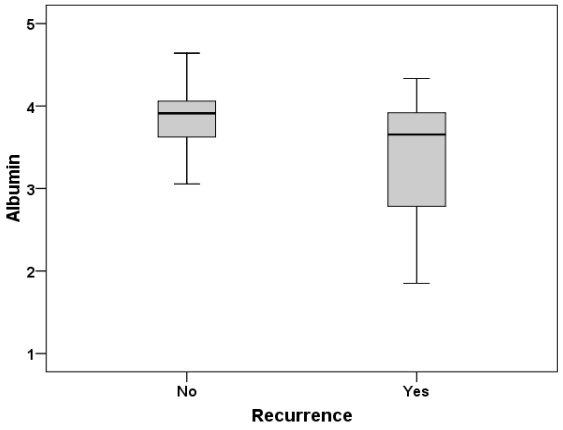

Figure 5.5 - Plot of the distribution of Albumin values according to the ocurrence of CRC recurrence.

In the current study, albumin values are significantly lower in the patients who experience recurrence in the inter-group analysis (Table 4.4 and Figure 5.5). However, albumin failed to provide significant prognostic information in the ROC curve analysis (Table 4.9).

Albumin has been considered as a pre-surgical prognostic factor. The mobilization of proteins for acute-phase synthesis, leading to a cachectic state in the establishment of an inflammatory response appears to be reasonable. Since most patients are only diagnosed with CRC at a somewhat advanced stage, the cachectic syndrome has had time to settle and slowly deplete the body's protein reserve. In the case of recurrent patients, they are more alert to the dangers of malnourishment and are alert for signs of the disease. They are also monitored more frequently, providing opportunity for correcting transient deficiencies in proteins.

5.2.5.3. Total White Blood Cells

The count of Total White Blood Cells is used to check if an inflammatory process is in place or an infection is present. In the context of rectal cancer, it has been found to be increased in advanced stages of the disease (Janisch *et al.*, 1994;Shoenfeld *et al.*, 1986) and terminal patients (Ventafridda *et al.*, 1991), related with shorter survival (Maltoni *et al.*, 1997) and significantly correlated with the presence of metastases in several malignancies, including colorectal However, metastasis was not an obligatory requirement for the presence leukocytosis (Shoenfeld *et al.*, 1986).

The pathophysiological processes that underlie the changes in TWBC counts are not clear. The elevation may be a result of direct or indirect processes such as (a) necrosis or inflammation or (b) effects on bone marrow or peripheral TWBC pools, respectively (Maltoni *et al.*, 1997).

In the present study, the white blood cell count was significantly higher in patients that experienced recurrences (Table 4.4 and Figure 5.6).

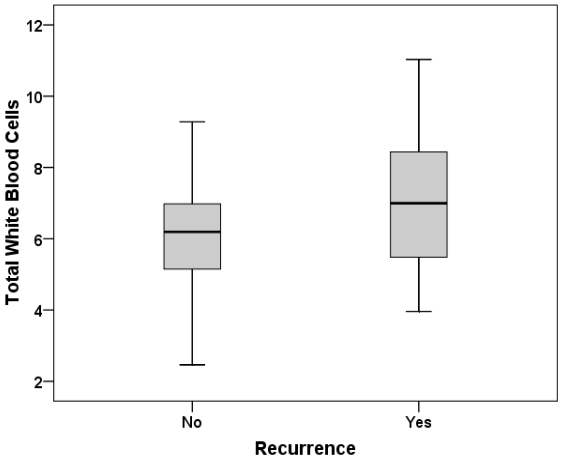

Figure 5.6 - Plot of the distribution of TWBC values according to the ocurrence of CRC recurrence.

TWBC's predictive capability was the third best in the ROC Curve analysis and patients with values above $6.8x10^3/\mu L$ were found at an increased risk of recurrence of about two times (Table 4.10). However, when included in a multivariate model, the remaining parameters were found more powerful in the prediction of recurrences (Table 4.11 and Table 4.12). The explanation provided in Section 5.2.1, concerning the characteristics of the statistical method employed, may also contribute to this loss of capability and the data must be interpreted with caution.

These findings need an extra-careful interpretation because the cut-off found in the ROC Curve analysis is well within the reference range for this parameter, $11.10x10^3/\mu L$, and a patient with 6.8 cannot be considered at greater risk of recurrence because it is a normal TWBC value. However, these results may point to a tendency towards higher values being related with recurrence, what would be in line with the results obtained in the aforementioned studies.

5.3. PRE-OPERATIVE DATA

In the ideal scenario, it would be possible to know, based on clinicopathological and biochemical data, if a patient is at high risk of developing a recurrence even before the curative resection of the primary tumor takes place. To that end, the parameters that had given promising signs in the early post-operative analysis were tested in the pre-surgical data, with negative results.

The only parameter that presented diagnostic capability was Alkaline Phosphatase in the ROC Curve analysis. Unfortunately, this parameter did not reach statistical significance in the Cox

proportional hazards model, proving that patients with elevated ALP are not at an increased risk of developing CRC recurrences.

The pre-operative database was even further reduced than the post-operative because, for many patients, that information was not readily available from their records in either format, paper or electronic. Additionally, an already reduced pool of 151 patients was reduced to 107, with only 17 recurrences and an even more reduced amount of data on each parameter (Table 4.15). This will have no doubt contributed to the negative results and only a more powerful study on these parameters will have the ability to study it thoroughly.

CHAPTER 6: CONCLUSIONS

Colorectal cancer is one of the leading causes of death of the modern world. The death rate is exceedingly high in advanced stages and even if detected in an early phase and potentially curative resection is performed, 40 to 50% of the patients will develop recurrence or metastatic disease (Kievit, 2002). The recurrence risk must be characterized as thoroughly as possible in order to identify that subset of patients and assign them to the best possible therapeutic plan.

Although there are markers already identified for recurrence like TNM Staging and Carcinoembryonic Antigen, they are still unable to predict it with precision. Consequently, all the sources of extra input on recurrence can be of great interest to patient management.

Although largely underpowered by the scarce number of patients with recurrences and deaths, it was possible to produce reliable results as can be seen by the conformity of the data on CEA and CA 19-9 with the literature.

The strongest predictor found in the analysis, aside from CEA, was blood glucose. The data on this parameter in colorectal cancer is conflicting and this study approached the subject from a different angle of the ones before it. Nevertheless, the rationale makes sense with the data, even though the difference between the values of the patients with and without recurrences is slight, but coherent through all the analyses.

C-Reactive Protein is a possible predictor of recurrence since it came very close to statistical significance in this work. A study with a larger sample will probably be able to shed more light on this parameter.

TWBC has to be further investigated, but the tendency towards a relation between high TWBC values and recurrence is signaled here.

Albumin values did not provide information on this work and the literature only considers its role on the preoperative period. The parameter appears to be of no use in the prediction of recurrences.

Liver function tests did not provide useful input as predictors although they are empirically used in the clinic since there is a logical relation between the possibility of a patient harboring liver

metastases and raised hepatic tests. Maybe Gamma-GT is in fact useful in that scenario but this work could not evaluate it properly.

Lactate Dehydrogenase, not being liver specific but frequently associated with its function, provided significant results that must be taken into account even though they were only useful if CEA was not considered in this analysis. The sample limitations do not allow an extrapolation that clearly states that it will only be useful if CEA is not measured.

Given the inherent limitations, the results are merely indicative, with very large confidence intervals. Nevertheless, the hint that blood glucose and perhaps lactate dehydrogenase may contribute to the assessment of recurrence risk along with CEA is an important result that invites confirmation by future, more powerful studies.

Studies of this nature are ideally performed on clinical data that has been registered under clear rules and organization. Collecting and organizing data from the daily clinical registries, in both paper and electronic formats, that was reported without standard reporting rules tends to be a difficult job. The results obtained in this study prove that it is not absolutely necessary to have the ideal registries to produce relevant results in health and expand scientific knowledge, walking towards improved medical care and that this is a possible repository of knowledge worth exploring.

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APPENDIX I

Table I-1 - Descriptive statistics of post-surgical biochemical data

	n				
	Valid	**Missing**	**Median**	**Minimum**	**Maximum**
Erythrocytes	2318	113	4.1	1.8	6.5
Hemoglobin	2318	113	12.4	6.1	17.5
Hematocrit	2318	113	37.7	17.6	54.7
Mean Corpuscular Volume	2318	113	92.0	56.0	121.0
Mean Corpuscular Hemoglobin	2318	113	30.4	16.0	40.8
MCHC	2317	114	33.0	25.4	90.8
RDW	2283	148	13.6	9.9	31.5
Total White Blood Cells	2317	114	6.2	0.3	52.4
Neutrophils	2315	116	3.8	0.0	46.7
Eosinophils	2313	118	0.1	0.0	2.1
Basophils	2313	118	0.0	0.0	1.0
Lymphocytes	2313	118	1.5	0.0	7.7
Monocytes	2312	119	0.4	0.0	9.8
Platelets	2305	126	213.0	6.0	1291.0
Erythrocyte Sedimentation Rate	184	2247	18.0	2.0	128.0
Prothrombin Time	630	1801	12.3	9.6	53.6
International Normalized Ratio	628	1803	1.0	0.0	4.8
Thromboplastin Time	564	1867	28.9	12.4	155.0
Glucose	2021	410	107.9	51.7	494.0
Blood Urea Nitrogen	2005	426	35.4	0.8	261.7
Creatinine	2005	426	0.9	0.2	60.4
Sodium	1956	475	139.3	101.0	170.1
Potassium	1955	476	4.3	2.1	6.5
Chloride	1948	483	104.0	76.0	139.0
Calcium	747	1684	8.9	3.1	10.7
Phosphorus	353	2078	2.9	0.5	10.1
Magnesium	530	1901	2.0	1.0	4.2
Creatine Phosphokinase	134	2297	81.5	8.0	1257.0
Aspartate Aminotransferase	1526	905	22.0	6.0	377.0
Alanine Aminotransferase	1521	910	30.0	3.0	440.0
Lactate Dehydrogenase	1297	1134	194.0	32.0	2799.0
Bilirubin - Total	1195	1236	0.6	0.1	10.0
Bilirubin - Direct	1095	1336	0.1	0.0	5.7
Bilirubin - Indirect	1094	1337	0.5	0.0	4.3
Alkaline Phosphatase	1335	1096	91.0	25.0	2037.0
Gamma-GT	1149	1282	37.0	4.0	1336.0
C-Reactive Protein	447	1984	4.1	0.0	33.8
Total Proteins	431	2000	7.0	2.7	8.6
Albumin	490	1941	3.8	0.7	5.2
Uric Acid	247	2184	4.9	1.9	10.3

	n				
	Valid	**Missing**	**Median**	**Minimum**	**Maximum**
Cholesterol - Total	177	2254	198.0	7.0	371.0
Cholesterol - HDL	155	2276	49.6	6.9	271.0
Cholesterol - LDL	138	2293	128.5	8.0	253.0
Triglycerides	162	2269	110.0	32.0	527.0
Amylase	114	2317	51.0	8.0	479.0
Lipase	116	2315	64.5	1.0	2013.0
Iron	119	2312	80.0	18.0	219.4
Ferritin	84	2347	70.0	4.0	1253.0
Alpha-Fetoprotein	136	2295	1.9	0.9	20.5
CEA	1019	1412	1.4	0.0	823.0
CA 19-9	985	1446	10.8	1.2	462.9
PSA	85	2346	0.9	0.2	4172.0

Table I-2 - Correlation coefficient of biochemical variables with CEA and CA 19-9.

	Spearman Correlation Coefficient	
	CEA (*p*)	**CA 19-9 (*p*)**
CEA	---	0.281 (<0.0001)
CA 19-9	0.281 (<0.0001)	---
Erythrocytes	-0.102 (0.001)	-0.033 (0.306)
Hemoglobin	-0.096 (0.002)	-0.021 (0.516)
Hematocrit	-0.07 (0.027)	-0.02 (0.532)
Mean Corpuscular Volume	0.118 (<0.001)	0.081 (0.012)
Mean Corpuscular Hemoglobin	0.061 (0.055)	0.094 (0.003)
MCHC	-0.134 (<0.001)	0.015 (0.645)
RDW	0.095 (0.003)	-0.035 (0.28)
Total White Blood Cells	0.138 (<0.001)	0.074 (0.022)
Neutrophils	0.123 (<0.001)	0.109 (0.001)
Eosinophils	0.08 (0.012)	-0.049 (0.131)
Basophils	-0.07 (0.028)	0.008 (0.816)
Lymphocytes	-0.023 (0.475)	-0.043 (0.187)
Monocytes	0.203 (<0.001)	0.083 (0.01)
Platelets	0.062 (0.051)	0.154 (<0.001)
Erythrocyte Sedimentation Rate	-0.017 (0.837)	0.016 (0.849)
Prothrombin Time	0.047 (0.677)	0.135 (0.235)
International Normalized Ratio	0.06 (0.591)	0.168 (0.139)
Thromboplastin Time	0.248 (0.031)	-0.001 (0.993)
Glucose	0.192 (<0.001)	0.125 (<0.001)
Blood Urea Nitrogen	-0.094 (0.005)	0.008 (0.825)
Creatinine	-0.025 (0.455)	0.067 (0.051)
Sodium	-0.133 (<0.001)	-0.11 (0.002)
Potassium	-0.072 (0.038)	-0.076 (0.032)
Chloride	-0.016 (0.654)	-0.12 (0.001)
Calcium	-0.063 (0.186)	-0.196 (<0.001)
Phosphorus	-0.054 (0.421)	0.188 (0.005)
Magnesium	-0.211 (<0.001)	-0.059 (0.283)
Creatine Phosphokinase	0.392 (0.233)	-0.119 (0.713)
Aspartate Aminotransferase	0.2 (<0.001)	0.114 (0.001)
Alanine Aminotransferase	-0.066 (0.053)	0.029 (0.402)
Lactate Dehydrogenase	0.356 (<0.001)	0.179 (<0.001)
Bilirubin – Total	0.098 (0.009)	-0.025 (0.524)
Bilirubin – Direct	0.119 (0.003)	0.069 (0.092)
Bilirubin – Indirect	0.086 (0.031)	-0.063 (0.123)
Alkaline Phosphatase	0.038 (0.281)	0.223 (<0.001)
Gamma-Glutamyltransferase	0.196 (<0.001)	0.284 (<0.001)
C-Reactive Protein	0.294 (0.005)	0.013 (0.906)
Total Proteins	-0.174 (0.004)	<0.001 (0.998)
Albumin	-0.119 (0.039)	-0.16 (0.006)
Uric Acid	0.16 (0.022)	0.181 (0.01)
Cholesterol – Total	0.35 (<0.001)	0.146 (0.113)

	Spearman Correlation Coefficient	
	CEA (*p*)	**CA 19-9 (*p*)**
Cholesterol – HDL	0.037 (0.705)	0.2 (0.037)
Cholesterol – LDL	0.407 (<0.001)	0.06 (0.546)
Triglycerides	0.287 (0.002)	0.382 (<0.001)
Amylase	0.203 (0.7)	0.071 (0.879)
Lipase	-0.036 (0.939)	0.214 (0.645)
Iron	-0.026 (0.79)	0.059 (0.553)
Ferritin	0.159 (0.176)	0.317 (0.006)
Alpha-Fetoprotein	-0.214 (0.014)	0.172 (0.051)
PSA	0.199 (0.116)	-0.141 (0.266)

Table I-3 - Mann-Whitney U tests of biochemical variables across groups of patients with and without recurrence.

	Recurrence		
	No	**Yes**	***p-value***
Erythrocytes	124	28	0.003
Hemoglobin	124	28	0.004
Hematocrit	124	28	0.002
Mean Corpuscular Volume	124	28	0.676
Mean Corpuscular Hemoglobin	124	28	0.748
Mean Corpuscular Hemoglobin Concentration	124	28	0.788
Red Blood Cell Distribution Width	124	28	0.621
Total White Blood Cells	124	28	0.022
Neutrophils	124	28	0.007
Eosinophils	124	28	0.395
Basophils	124	28	0.572
Lymphocytes	124	28	0.044
Monocytes	124	28	0.021
Platelets	124	28	0.266
Erythrocyte Sedimentation Rate	17	2	0.595
Prothrombin Time	87	24	0.443
International Normalized Ratio	87	24	0.559
Thromboplastin Time	77	22	0.920
Glucose	124	28	0.010
Blood Urea Nitrogen	124	28	0.951
Creatinine	124	28	0.260
Sodium	123	28	0.485
Potassium	123	28	0.041
Chloride	123	28	0.444
Calcium	78	25	0.633
Phosphorus	51	23	0.843
Magnesium	62	24	0.331
Creatine Phosphokinase	21	11	0.858
Aspartate Aminotransferase	110	28	0.010
Alanine Aminotransferase	110	28	0.571
Lactate Dehydrogenase	102	28	<0.001
Bilirubin - Total	100	27	0.920
Bilirubin - Direct	96	27	0.085
Bilirubin - Indirect	95	27	0.476
Alkaline Phosphatase	105	28	0.014
Gamma-GT	99	26	0.048
C-Reactive Protein	51	19	0.031
Total Proteins	56	21	0.075
Albumin	60	21	0.034
Uric Acid	37	7	0.810
Cholesterol - Total	31	3	0.316
Cholesterol - HDL	27	3	0.090
Cholesterol - LDL	22	2	0.047

	Recurrence		
	No	**Yes**	***p-value***
Triglycerides	29	3	0.146
Amylase	11	8	0.901
Lipase	14	8	0.041
Iron	24	2	0.124
Ferritin	20	1	0.620
Alpha-Fetoprotein	17	6	0.183
CEA	113	23	<0.0001
CA 19-9	114	23	<0.0001
PSA	11	2	0.324

Table I-4 – Descriptive statistics of pre-surgical biochemical data

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	Valid	**Missing**	**Median**	**Minimum**	**Maximum**
Erythrocytes	107	0	4.4	3.4	5.9
Hemoglobin	107	0	12.6	8.1	24.6
Hematocrit	107	0	38.4	27.7	50.7
Mean Corpuscular Volume	107	0	88.8	57.7	100.3
Mean Corpuscular Hemoglobin	107	0	29.4	17.2	33.2
MCHC	107	0	32.5	29.1	35.1
RDW	101	6	14.3	11.3	24.1
Total White Blood Cells	107	0	7.0	3.1	19.8
Neutrophils	106	1	4.5	1.3	16.2
Eosinophils	106	1	0.2	0.0	0.8
Basophils	106	1	0.0	0.0	0.1
Lymphocytes	106	1	1.7	0.1	7.0
Monocytes	106	1	0.5	0.2	2.3
Platelets	101	6	271.5	64.4	598.6
Erythrocyte Sedimentation Rate	21	86	27.5	5.5	51.5
Prothrombin Time	83	24	11.8	10.1	18.7
International Normalized Ratio	83	24	1.0	0.8	1.6
Thromboplastin Time	72	35	27.1	21.8	45.9
Glucose	97	10	105.2	75.6	322.7
Blood Urea Nitrogen	93	14	38.1	12.0	118.9
Creatinine	94	13	0.9	0.6	2.0
Sodium	89	18	139.5	120.9	152.0
Potassium	89	18	4.4	3.5	5.8
Chloride	89	18	103.3	96.5	124.2
Calcium	7	100	6.5	4.8	9.4
Phosphorus	2	105	1.4	1.2	1.6
Magnesium	2	105	2.1	1.9	2.3
Creatine Phosphokinase	14	93	45.8	18.0	144.5
Aspartate Aminotransferase	72	35	20.5	6.5	144.5
Alanine Aminotransferase	70	37	21.9	10.0	157.1
Lactate Dehydrogenase	42	65	179.2	114.0	303.5
Bilirubin - Total	49	58	0.7	0.1	1.6
Bilirubin - Direct	41	66	0.1	0.1	0.7
Bilirubin - Indirect	41	66	0.5	0.3	1.1
Alkaline Phosphatase	48	59	75.0	43.0	196.8
Gamma-GT	42	65	24.8	8.5	204.0
C-Reactive Protein	41	66	2.2	0.1	22.6
Total Proteins	20	87	6.8	5.9	7.7
Albumin	24	83	3.9	3.0	4.7
Uric Acid	18	89	5.2	3.0	7.5
Cholesterol - Total	15	92	215.0	135.4	265.0
Cholesterol - HDL	14	93	48.1	30.5	65.0
Cholesterol - LDL	12	95	128.8	80.0	175.0

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	Valid	**Missing**	**Median**	**Minimum**	**Maximum**
Triglycerides	14	93	137.3	56.0	171.0
Amylase	15	92	43.5	24.5	572.8
Lipase	14	93	82.5	6.0	1846.0
Iron	6	101	17.9	11.5	31.2
Ferritin	7	100	20.0	7.5	261.9
Alpha-Fetoprotein	6	101	1.5	1.0	2.6
CEA	22	85	2.4	0.5	176.2
CA 19-9	18	89	10.2	2.5	105.7
PSA	10	97	1.2	0.2	6.4